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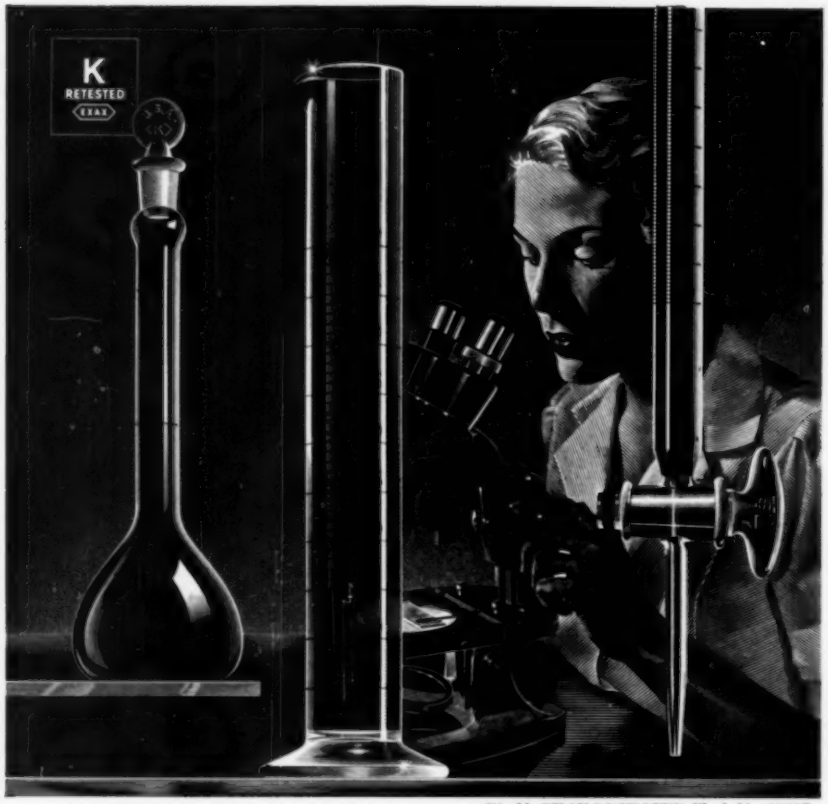

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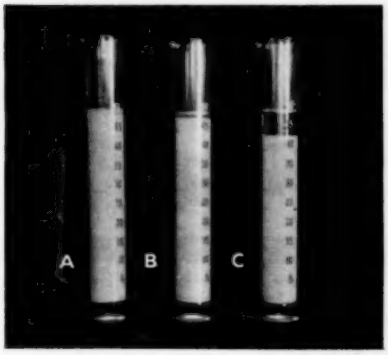

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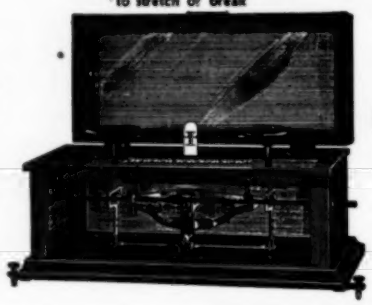

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Biological Effects of Salts of Ethylenediamine Tetra-acetic Acid*

By ROBERT BRENDEL, VINTON SWAYNE, ROBERT PRESTON, J. M. BEILER, and GUSTAV J. MARTIN

ETHYLENEDIAMINE tetra-acetic acid (Sequestrene, Trilon B) is known to function as a powerful chelating agent (1, 2). The relatively easy solubility and low toxicity of this compound have made it of interest biologically, since many of the reactions occurring in the body are known to be influenced by metallic ions. It has been reported to inhibit the oxidation of ascorbic acid by the formation of a copper complex (3), and to function as a solvent for urinary calculi of certain types (4, 10). Of particular interest have been the reports of the effectiveness of the compound as an anticoagulant *in vitro;* yet it has been found not to have this property in the body (5–8). This is surprising, inasmuch as the anticoagulant effect has been reported to be due to chelation of calcium, and ethylenediamine tetra-acetate has been found actually to lower serum calcium *in vivo* (9).

All these reports suggested the desirability of a more detailed study of the effects of the compound in the form of different salts in order to determine whether it would function as an effective chelating agent in the body and whether it could be used as a vehicle for the transport of metallic ions.

* Received January 23, 1952, from the Research Laboratories, The National Drug Co., Philadelphia, Pa.

EXPERIMENTAL

A number of salts of ethylenediamine tetra-acetic acid were tested to determine their effect on blood coagulation *in vitro.* It has been shown that the anticoagulant action of the disodium salt is due to chelation of calcium; the results of this test therefore provided an indication of the chelating powers of the different salts under the conditions of the experiment. Dog blood was used throughout. The compounds were added to the blood in saline, 0.1 cc. to 1 cc. of blood. Saline alone was used in the controls.

Toxicity tests were run on the series of salts to determine whether there was any relationship between the chelating properties of the compounds as manifested by their effects on coagulation *in vitro* and their effects in the body as manifested by toxicity. Mice weighing 20–26 Gm. were used for this work, with the compounds being injected intraperitoneally in aqueous medium.

RESULTS AND DISCUSSION

The disodium salt of ethylenediamine tetra-acetate was chosen as a standard of comparison because of the great number of reports on this compound already in the literature. It is an effective anticoagulant *in vitro;* a typical set of results is presented in Table I.

The maximum effect occurs at 0.5 mg./cc.; the other salts tested were therefore run at this concentration. Results are given in Table II.

The results show that only the dipotassium salt had any effect on coagulation *in vitro.* Since the

TABLE I.—ANTICOAGULANT EFFECT OF DISODIUM ETHYLENEDIAMINE TETRA-ACETATE

Control Clotting Time	Disodium Ethylenediamine Tetra-acetate, Mg./Cc.	Disodium Ethylenediamine Tetra-acetate Time
3 min. 15 sec.	0.2	3 min. 15 sec.
3 min. 20 sec.	0.3	5 min. 5 sec.
5 min. 30 sec.	0.4	11 min. 5 sec.
5 min. 5 sec.	0.5	No coagulation

TABLE II.—ANTICOAGULANT EFFECTS OF VARIOUS SALTS OF ETHYLENEDIAMINE TETRA-ACETATE

Salt	Anticoagulant Effect
Dipotassium	No coagulation
Monomagnesium	None
Dimagnesium	Slight
Tetra-sodium	Slight
Ferrous	None
Monocalcium	None
Neodymium	None
Copper	None
Cobalt	None

coagulation effect is due to chelation with calcium, the interpretation is that only the dipotassium salt is capable in blood of exchanging its ions with calcium. This is in accord with the report of Martell and Bersworth (2) that the ionization of heavy metal salts of ethylenediamine tetra-acetate is almost completely suppressed, which would also mean that any heavy metal ions attached to the nucleus would remain so in the body and that the ions would not become available to the body as such. It is of interest to note that the tetra-sodium salt was relatively ineffective as an anticoagulant. Whether a suppression of ionization is involved in this case is not known.

As has been stated, the difference between the anticoagulation effects of the disodium salt of ethylenediamine tetra-acetic acid *in vitro* and *in vivo* would indicate that chelation does not occur in the body, or at least does not occur to the same extent as it does *in vitro*. The fact that the heavy metal salts had been found to have no effect on coagulation *in vitro* suggested that a comparison of some of the *in vivo* effects of these compounds with those of the disodium salt might indicate whether any of the effects of the latter were due to chelation. Toxicity was chosen as a critical test. Results are presented in Table III.

There is no significant difference between most of the figures obtained and those for the disodium salt. In the few cases where the LD_{50} is appreciably higher, it seems probable that poor solubility and slow absorption are involved. In all cases the toxicity symptoms were the same as those found for the disodium salt; convulsions were produced and death resulted from asphyxia.

TABLE III.—TOXICITIES OF VARIOUS SALTS OF ETHYLENEDIAMINE TETRA-ACETATE

Salt	LD_{50}, Mg./Kg.
Disodium	400
Monomagnesium	500
Tetra-sodium	350
Ferrous	350
Dimagnesium	450
Dipotassium	450
Monocalcium	500
Neodymium	1,000
Copper	600
Cobalt	1,000

It would therefore appear that at least acute toxicity and the pharmacological manifestations connected with it are not associated with chelation on the ethylenediamine tetra-acetate molecule. The same toxicities have been shown to be associated with different salts regardless of their *in vitro* chelating properties. The fact that the *in vitro* chelating action of the disodium salt, as measured by its anticoagulant effect, is not carried over into the body is further evidence of this fact. The compounds do not seem to function as chelating agents in the body, at least in acute tests.

SUMMARY

A series of metallic salts of the ethylenediamine tetra-acetic acid were tested both *in vivo* and *in vitro*. Some of the compounds were found to function as chelating agents *in vitro;* there was no evidence of such activity in the body.

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The Detection of Intracranial Lesions, Especially Recurrent Tumors, by the Use of Radioactive Isotopes and the Scintillation Counter*

By M. ASHKENAZY†

WITH THE USE of a more stable radioactive dye, diiodofluorescein, a progressive modification of the technique of external counting by employing an automatic graphic recorder and count-rate meter, and, lately, the use of a scintillation counter, an accuracy of approximately 90 per cent has been obtained in the localization of brain tumors. The recent acquisition of a newer radioactive isotope which is not a vital dye but a normal component of human serum, i. e., radioactive iodinated human serum albumin, has added greatly to the possibilities of this test.

We have further extended and improved the technique of the radiodye test by the use of a modified scintillation counter which is 100 to 150 times more sensitive than the conventional Geiger-Mueller tube, or even the newer bismuth cathode tubes. Because of the greater precision and sensitivity of this scintillation counter, not only can the dose of radioactive isotopes be reduced, but actual testing time can be reduced to twenty to forty minutes, as contrasted to two and one-half to four hours required by conventional G-M tubes and one and one-half hours by bismuth cathode tubes.

This test is particularly accurate in the diagnosis of early tumor recurrences. Other neurosurgical diagnostic procedures are very fallacious in this respect, for pneumograms always show distortions of the ventricular pattern which is the result of the operative defect and the secondary gliosis and postoperative fibrosis. For the same reason, angiography will show abnormal vascular patterns postoperatively, and electroencephalography will also show abnormal electrical patterns which are difficult to interpret from findings in cases of early tumor recurrences. Clinically the difficulty may be even greater. For example, in the differential diagnosis of postoperative focal epilepsy, it is difficult to decide whether this is due to gliovascular changes secondary to surgery or to an early tumor recurrence.

This feature assumes vast importance in those benign tumors whose removal results in prolonged survival time for the patient, for the more complete and earlier the removal of a recurrent benign tumor, the better the eventual prognosis. Follow-up studies now take only fifteen to twenty minutes and their results have correlated extremely closely with the degree of completeness or incompleteness of removal of the tumor at surgery.

These tests have already proved to be of great value in services other than neurosurgical, e. g., to thoracic surgeons in their decisions to perform radical pneumonectomy or lobectomy for primary bronchogenic carcinoma. By scanning the skull following the administration of either radioactive diiodofluorescein or iodinated human serum albumin, we have been able to rule out metastatic lesions to the brain which, when present, would of course obviate the necessity for such extensive surgery. These metastatic cerebral lesions have usually been detected by this method much earlier than by any of our present clinical means of detection.

Again it was noticed in these more recent studies with newer radioactive isotopes that the affinity of the isotope for tumor tissue is related to the cellularity and vascularity of the neoplasm with the more malignant ones showing the greater degree of concentration. Metastatic carcinomas and glioblastomas give counts in the highest differential range with the more cellular meningiomas following closely behind. The oligodendrogliomas and astrocytomas show the lowest radiodye concentrations, coinciding directly with the microscopic scarcity of capillaries in such tumors. Large cysts and cystic degenerative lesions may yield even lower than normal readings since only the active cellular and vascular tumor tissue concentrates the radiodye.

Again, localization was much more focally accurate than with air studies, electroencephalography, or angiography, and there was no mortality nor morbidity. An interesting feature is that the utilization of this radioisotope test combined with the findings of any one of the other older neurosurgical diagnostic procedures yields almost 100 per cent accuracy in diagnosis. This

* Received August 4, 1952, from the Veterans Administration Hospital, Houston, Tex.

Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952, by Dr. Moses Ashkenazy, co-recipient of the fourth (1951) Chilean Iodine Educational Bureau, Inc., Award for outstanding research in the chemistry and pharmacy of iodine.

† Chief, Neurosurgery, Veterans Administration Hospital, Houston.

is particularly true if all other tests are combined, such as clinical acumen, electroencephalography, or pneumoencephalography.

Air studies with their severe headaches and occasional shock are markedly discomforting procedures so far as the patient is concerned, in contrast to the relative harmlessness and simplicity of the radiodye test. Ventriculography in itself, though of extreme accuracy, is not entirely a harmless procedure, and a certain percentage of deaths from this cause alone is recorded in all neurosurgical clinics, especially in those patients with markedly increased intracranial pressure.

The selective deposition of the radiodye or larger colloid particles within tumor tissue appears to be dependent chiefly upon the state of the capillary endothelium, and these concentrations are directly proportional to the histopathological patterns of the blood vessels, particularly the terminal capillaries as visualized under the microscope. Previous experimental studies have also proved that changes in endothelial permeability permit the exudation of foreign substances as well as vital dyes and colloids. Our studies on mice, dogs, and human biopsy and radio assay materials tend to confirm these older observations.

Just as the radiologist is acquainted with the normal variations in the ventricular pattern, or the angiographic patterns of the brain, so the isotopologist must be aware of the normal range of radiodye concentrations at specific time intervals for specific areas of the brain. Each of the thirty-two locations over the skull normally vary in their radiodye concentrations at the same interval of time following isotope administration.

In distinction to other neurosurgical diagnostic tests, such as angiography, there have been no contraindications with regard to advanced age, very advanced arteriosclerosis, recent thrombotic or embolic episodes, acute intracranial hemorrhage, hypertension, or cardiac decompensation.

The radiodye test does not differentiate an acute infection from an anaplastic tumor, since the disturbed capillary permeability in both types of lesions will result in an increased exudation of dye. In a cystic degenerative tumor, only the actively growing tumor tissue concentrates the radioactive material, whereas the cystic portions actually have a diminished uptake. When the preponderance of cyst fluid over tumor cells and tumor capillaries is too great, the uptake is not significantly increased, thereby giving rise to most of the errors expected to be encountered in this method of diagnosis.

Isotope Encephalometry: External Localization Techniques for the Diagnosis of Brain Tumors*

By GEORGE E. MOORE†

As an interne in 1936, the author initiated a series of clinical studies in an attempt to find a better method of determining the site of amputation for gangrenous extremities. Sodium fluorescein was injected into the femerol artery and the fluorescent pattern of the leg was observed under ultraviolet light. In the course of these studies, a few patients being operated upon with the diagnosis of malignancy were injected with fluorescein and their abdomens examined with ultraviolet light at the time of operation. Although the first few patients showed no evidence of localization of the fluorescent dye in the tumor tissue, one individual with a mucinous carcinoma of the stomach did show brilliant fluorescence of the primary tumor and the peritoneal metastases present. Unfortunately, it was found that not only malignant tissue fluoresced, but also areas of inflammation; therefore, the clinical usefulness of the test was minimized.

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Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952, by Dr. George E. Moore, co-recipient of the fourth (1951) Chilean Iodine Educational Bureau Award for outstanding research in the chemistry and pharmacy of iodine.

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A series of patients with brain tumors was also subjected to the fluorescein test, and in contrast to the indifferent results obtained with abdominal malignancies by this technique, the test seemed to be quite accurate in identifying brain tumors (1). It should be pointed out, however, that it was soon apparent that the fluorescein test was not specific for brain tumors alone, but also was present in other lesions of the central nervous system in which there was a disturbance of the so-called blood-brain-barrier which prevents the entrance of toxic materials into the brain.

Normally the central nervous system is protected from the entrance of negatively charged molecules by the so-called blood-brain-barrier mechanism (BBB). This protective barrier is present throughout the central nervous system with the notable exception of the coroid plexus and several other areas. In the presence of brain edema, or acute inflammation, the protective barrier (BBB) is disrupted, and in these areas the negatively charged materials can flood into the brain substance. The newly developing vessels of brain tumors also do not have this protective mechanism, and thus they allow certain dyes to accumulate in the tumor tissue. In addition there is an undetermined factor of specific protein binding of the dye by tumor cell contents.

The fluorescein technique is useful at operation since the neurosurgeon can aspirate a small bit of tissue deep in the brain, examine it under the ultraviolet light, and, if fluorescent material is present, be reasonably sure that he has encountered a brain tumor. It may also be of help in determining the extent of the tumor. To date, this technique has been employed with more than 500 patients at the University of Minnesota Hospital.

Following the success of the fluorescein technique, many attempts were made to synthesize radiopaque fluorescein derivatives in the hope of finding a substance which would be taken up in the brain tumor sufficiently to allow diagnosis of the tumor by ordinary roentgenographic methods. These compounds included iodinated fluoresceins containing from two to eight atoms of iodine per molecule. Unfortunately, however, with the increasing addition of iodine to the molecule, the lessening of solubility and increase in toxicity of these compounds mitigated against their usefulness.

The failure to develop radiopaque compounds which could be used clinically stimulated investigation of the use of radioactive ions which would tag the fluorescein and make possible identification of concentrations of the dye and, therefore, localization of brain tumors before operation. The most promising of the tracers was diiodofluorescein.[1]

In 1948, the successful use of radioactive diiodofluorescein for the preoperative diagnosis of brain tumors was reported (2). In brief, the radioactive dye was injected intravenously, and some hours later a sensitive, shielded Geiger-Mueller counter was employed to seek unusual concentrations of radioactivity emanating from the head (Fig. 1). Asymmetrical concentrations of radioactivity were found to indicate the presence of a brain tumor. Table I illustrates the results of such a test. Similarly, patients suspected of having a brain tumor in which no localization of activity could be found usually had a cerebral vascular accident or some other nonmalignant neurological disorder. Interestingly enough, the success of localizing brain tumors was found to parallel the degree of malignancy. Most difficult was the localization of slow-growing types of tumors; on the other hand, tumors such as glioblastomas were found readily. Dissatisfaction with the brief interval during which the patients could be counted and resurveyed, and the inability to use techniques other than simple comparison of a single set of symmetrical positions stimulated a search for more efficient radioactive tracers.

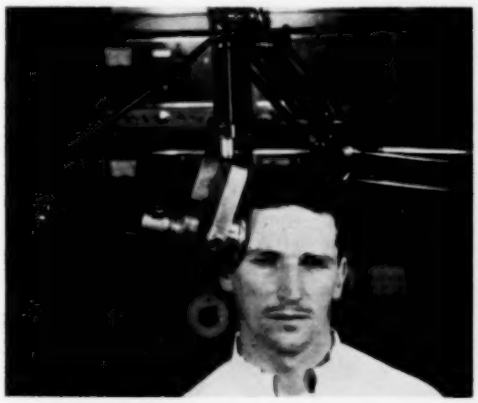

Fig. 1.—This patient has received an intravenous injection of sodium diiodofluorescein one hour previously. The head is surveyed for sites of greater radioactivity by "counting" the gamma emission from symmetrical areas. The instrument depicted is a scintillation counter utilizing an anthrocene crystal and an R. C. A. #5819 photomultiplier tube.

[1] This compound was later purified by Tabern of Abbott Laboratories.

TABLE I

Hosp. No. 83774 Name R. K. Age 47 Wt. 109 Date 10-31-51
Injection Time: 8:50 a. m. Tracer: Iodine (tagged Human Serum Albumin)
Counting Started: 8:30 a. m. Dosage: 250 μc. Counter: Scintillation

Position	C. P. M.	Differential Counting Rate	Re-Check C. P. M.	Re-Check Differential Counting Rate
Right supraorbital	8133	R-17.1%	8186	R-18.4%
Left supraorbital	6766	...	6670	...
Right lateral zygoma	8897	R-2.5%	...	...
Left lateral zygoma	8654	...	...	...
Right lateral frontal	7403	R-13.0%	7362	R-17.6%
Left lateral frontal	6449	...	6065	...
Right top frontal	7686	R-9.3%	7587	R-6.6%
Left top frontal	6954	...	7047	...
Right external ear	8774	R-3.1%	...	...
Left external ear	8496	...	...	...
Right lateral parietal	6849	R-1.7%	...	...
Left lateral parietal	6947	...	...	...
Right top parietal	7497	R-6.1%	...	...
Left top parietal	7046	...	...	...
Right occipital	7543	...	...	...
Left occipital	7662	L-1.4%	...	...
Right lateral occipital	6902	R-1.3%	...	...
Left lateral occipital	6803	...	...	...
Right mid-parietal	7209	...	...	...
Left mid-parietal	7446	L-3.2%	...	...
Right mid-occipital	7489	...	...	...
Left mid-occipital	7503	L-0.2%	...	...
Right mid-occipital	7804	R-3.4%	...	...
Left mid-occipital	7546	...	...	...

Conclusion: Metastatic carcinoma, superficial, right frontal lobe.

Theoretical considerations pointed to the possibility that the radioactive fluorescein might be combining with one of the proteins in the blood. This led to the discovery that human serum albumen which was tagged with radioactive iodine also would effectively localize brain tumors. Radioactive albumin was found to give stable levels of activity, was easy to use, and gave larger differential concentrations of activity over the brain tumors. The one disadvantage of this technique is the release of radioactive iodine from the albumin molecule, thereby allowing free radioiodine to be picked up by the patient's thyroid gland. This occurrence can be prevented by saturating the patient's thyroid with stable iodine (Lugol's solution), given one day previous to the injection of the radioactive tracer. At the present time the radioactive iodine-tagged human-serum albumin is the tracer of choice for isotope encephalometry.

Recently, radioiodine (KI) itself has been used following blockage of the patient's thyroid by pretreatment with Lugol's solution. Apparently most of the radioiodine which is injected becomes combined loosely with the patient's serum albumin and thereby simulates the conditions found when the albumin and iodine have been combined previous to injection. However, the disadvantages of using free radioactive ions, such as possible thyroid injury and the instability of the activity levels obtained, has resulted in a discontinuance of this technique.

Radioactive potassium (K^{42}) has also been used for the diagnosis of brain tumors (3). After intravenous injection of the tracer, the head is surveyed with a Geiger-Mueller counter similar to the method described for the diiodofluorescein method. Interestingly enough, radioactive potassium localizes markedly in brain tumors. However, this technique is clinically disadvantageous because of the extremely short half-life of the isotope which makes frequent shipments of the isotopes necessary, and also because the shielding problems necessitated by its powerful gamma radiation magnify instrumentation problems.

One of the promising new techniques in this field is the use of positron-emitting isotopes (4). Since these isotopes send out simultaneous gamma rays which are given off at 180°, a method employing the principles of coincidence counting,

using one counter on each side of the head, can be accomplished. With this method, localization of an area of radioactivity can be more sharply defined. To date, difficulty has been encountered in finding effective tracers to carry the positron-emitting isotopes.

Brief mention of the parallel development of instrumentation for isotope encephalometry should be made. When the original G-M counters were employed, large doses of the radioactive dye had to be given to ensure adequate counting rates. Later, more efficient tubes such as the bismuth-cathode tube became available and the dosage could be reduced. The development of stable scintillation counters has further reduced the amount of radioactivity necessary.

At this clinic an instrument which fits over the head and contains eighteen counter units has been tested. However, until certain mathematical calculations can be solved, it offers little advantage over the use of a single scintillation counter (Fig. 1).

SUMMARY

The advent of isotope encephalometry has given neurosurgeons an additional adjunctive diagnostic method for the localization of the brain tumors. It is innocuous and can be carried out without the use of an operative procedure. The accuracy of localization obtained with this method approaches that of the other diagnostic techniques and, when interpreted with the neurological findings of the patient, results in a diagnostic accuracy of approximately 90 per cent.

It would seem that, in the future, tracer diagnostic agents with lesser biological limitations will be found for the localization of tumors not only in the brain but also in other parts of the body.

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The Synthesis of Azo Compounds for Urogenital Analgesia*

By ELIOT STEINBERG, GUY M. MATTSON, and GEORGE E. PHILLIPS†

Eight azo-type dyes and their hydrochlorides have been synthesized. These compounds have potential therapeutic use for analgesia of the genitourinary tract. The physical and chemical properties of these compounds are reported.

CERTAIN azo compounds have found clinical acceptance for the treatment of genitourinary infections. Two examples are 3-phenylazo-2,6-diaminopyridine monohydrochloride,[1] I (1) and *p*-ethoxyphenylazo-2′4′-diaminobenzene monohydrochloride,[2] II (2). The usefulness of these drugs is apparently due primarily to their analgesic action rather than to their relatively slight antiseptic potency (3).

Esters of *p*-aminobenzoic acid have been widely accepted for therapeutic use as analgesics and local anesthetics (4). Ethyl *p*-aminobenzoate, in particular, is satisfactory as a local anesthetic because of its low toxicity. It is also reported to be active as a bladder analgesic (5).

In an attempt to enhance the analgesic properties of compounds of the above type for bladder analgesia, azo dyes of types III and IV were prepared from alkyl esters and from dialkylaminoalkyl esters of *p*-aminobenzoic acid.

The alkyl esters of *p*-aminobenzoic acid, where not commercially available, were prepared by esterification of the acid with the appropriate alcohol saturated with dry hydrogen chloride gas (6). *n*-Propyl *p*-aminobenzoate and isopropyl *p*-aminobenzoate were prepared in this manner. Adams, *et al.* (7), prepared these esters by the reaction of *p*-nitrobenzoyl chloride with

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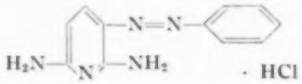
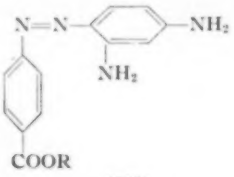
[1] Trademark name: "Pyridium" and "Mallophene."
[2] Trademark name: "Serenium."

the appropriate alcohol and the subsequent reduction of the nitro ester with iron and hydrochloric acid. The butyl *p*-aminobenzoate and N,N-dibutylaminopropyl *p*-aminobenzoate were supplied through the courtesy of the Special Chemicals Division of the Abbott Laboratories. The



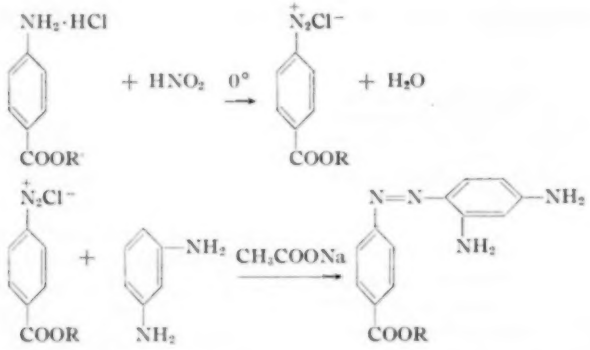
(I)

other *p*-aminobenzoates were obtained from the Matheson Co. and were used without further purification. The *m*-phenylenediamine, available in a technical grade, was recrystallized from benzene before using.



(III)

The diazotization and couplings were performed in the usual manner by diazotizing the local anesthetic and coupling it with *m*-phenylenediamine:

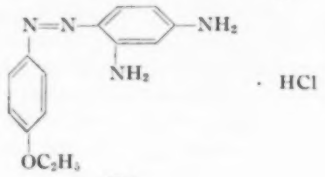


2-Diethylaminoethyl *p*-(2′,4′-diaminophenylazo)-benzoate and propyl *p*-(2′,4′-diaminophenylazo)-benzoate have previously been prepared for their histological staining properties (8). However, no experimental details, analytical data, or physical constants were reported.

Pharmacological studies of these compounds are in progress.

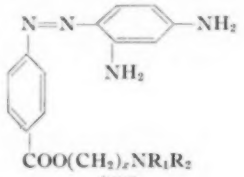
EXPERIMENTAL

***n*-Propyl *p*-Aminobenzoate.**—Sixty-nine grams (0.5 mole) of *p*-aminobenzoic acid was refluxed for forty-eight hours with 1 L. of *n*-propyl alcohol which had been saturated with dry hydrogen chloride gas. The resulting solution was diluted with an equal



(II)

volume of water and made basic with 20% sodium carbonate solution. An oil layer separated and slowly crystallized. The crystals, which were removed by filtration and recrystallized from hexyl ether, had a melting point of 73–76°; reported (7) 73–74°. A yield of 84.4 Gm. (93%) was obtained.



(IV)

Isopropyl *p*-aminobenzoate, prepared by the same procedure, had a melting point of 85–87°; reported (7), 85–86°.

Ethyl *p*-(2′,4′-Diaminophenylazo)-benzoate.—A quantity of 16.5 Gm. (0.1 mole) of ethyl *p*-aminobenzoate was dissolved in a solution of 200 cc. of water and 20 cc. of hydrochloric acid (sp. gr. 1.19). This solution was placed in an ice bath and diazotized with 1 *M* sodium nitrite solution. The solution of the diazonium salt was coupled with a solution of 10.8 Gm. (0.1 mole) of *m*-phenylene diamine and 42 Gm. (0.1 mole) of sodium acetate in 400 cc. of water. The resulting dark red solution was stirred for thirty minutes and then adjusted to a *p*H of 9 with ammonium hydroxide. The deep scarlet

TABLE I

Compd. No.	Compound Diazotized	R	Formula	M. P., °C.[a]	Color	Analysis, % N Calcd.	N Found	Cl Calcd.	Cl Found	Solubility, Gm./100 Cc. C_2H_5OH	Solubility, Gm./100 Cc. H_2O
1	Methyl *p*-aminobenzoate	CH_3	$C_{14}H_{14}N_4O_2$	201–203	Red	20.7	20.0	...	...	0.310	0.017
			$C_{14}H_{14}N_4O_2 \cdot HCl$	247–248	Red	18.5	18.3	11.6	11.5	0.188	0.038
2	Ethyl *p*-aminobenzoate (Benzocaine)	C_2H_5	$C_{15}H_{16}N_4O_2$	160–163	Scarlet	19.7	19.7	...	...	1.93	0.012
			$C_{15}H_{16}N_4O_2 \cdot HCl$	264–265	Maroon	17.5	16.8	11.1	10.9	0.100	0.044
3	Propyl *p*-aminobenzoate (Propaesin)	C_3H_7	$C_{16}H_{18}N_4O_2$	153–155	Scarlet	18.7	18.3	...	...	0.958	0.017
			$C_{16}H_{18}N_4O_2 \cdot HCl$	256–257	Red	16.7	16.2	10.8	10.2	0.744	0.017
4	Isopropyl *p*-aminobenzoate	*i*-C_3H_7	$C_{16}H_{18}N_4O_2$	138–140	Scarlet	18.7	18.7	...	...	2.98	0.010
			$C_{16}H_{18}N_4O_2 \cdot HCl$	245.5	Red	16.7	16.4	10.8	11.4	0.580	0.304
5	Butyl *p*-aminobenzoate (Butesin)[b]	C_4H_9	$C_{17}H_{20}N_4O_2$	106–110	Brown	17.9	17.2	..	...	6.98	0.016
			$C_{17}H_{20}N_4O_2 \cdot HCl$	248–249	Brown	16.1	15.2	10.2	9.7	0.790	0.050
6	Isobutyl *p*-aminobenzoate (Cycloform[c])	*i*-C_4H_9	$C_{17}H_{20}N_4O_2$	114–119	Maroon	17.9	17.3	...	...	6.39	0.009
			$C_{17}H_{20}N_4O_2 \cdot HCl$	228–229	Red	16.1	15.5	10.2	10.7	0.806	0.046
7	N,N-Diethylaminoethyl *p*-aminobenzoate (Procaine)	$(C_2H_5)_2NCH_2CH_2$	$C_{19}H_{25}N_3O_2$	107–109	Scarlet	19.7	19.3	...	...	18.12	0.078
			$C_{19}H_{25}N_3O_2 \cdot 2HCl$	236–237	Red	16.4	15.8	16.4	16.3	0.570	27.79
8	N,N-Dibutylaminoethyl *p*-aminobenzoate (Butacaine[b])	$(C_4H_9)_2NCH_2CH_2$	$C_{24}H_{35}N_3O_2$	101–104	Scarlet	16.5	16.4	...	...	5.34	00.38
			$C_{24}H_{35}N_3O_2 \cdot HCl$	246–248	Red	14.1	13.5	14.1	14.0	0.664	08.50

[a] Uncorrected.
[b] Trade mark: Abbott Laboratories.
[c] Trade mark: Winthrop-Stearns, Inc.

colored crystals were removed by filtration, washed with dilute ammonium hydroxide and water, and dried in a desiccator. The yield was almost quantitative. When recrystallized from absolute methanol, the base melted at 160–163°.

Anal.—Calcd. for $C_{18}H_{16}N_4O_2$: N, 19.7. Found: N, 19.7.

The hydrochloride was formed by dissolving the free base in ether and passing dry hydrogen chloride gas through the solution. The precipitated hydrochloride was filtered by suction and washed with absolute ethanol and ether. Dark red crystals which melted at 264–265° were obtained.

Anal.—Calcd. for $C_{18}H_{16}N_4O_2 \cdot HCl$: N, 17.5; Cl, 11.1. Found: N, 16.8; Cl, 10.9.

Compounds 1, 3, 4, 5, 6, 7, and 8 and their hydrochlorides were prepared in a similar manner and are described in Table I. It has also been found possible to isolate the hydrochloride directly by salting it out of the coupling solution before neutralization.

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Investigation of Some Complexes Formed in Solution by Caffeine*,†

II. Benzoic Acid and Benzoate Ion

By TAKERU HIGUCHI and D. A. ZUCK‡

The stoichiometry and the nature of interactions between caffeine and benzoic acid are discussed. Experimental evidence is offered to support the existence of at least one type of complex between caffeine and benzoate ion. The approximate stability constants for these systems have been determined at several temperatures to permit tentative estimation of the heats and entropies of the reactions.

THE present communication is concerned with the general tendency of caffeine to form soluble and insoluble complexes with a number of pharmaceuticals. In a previous report (1), evidence for the formation of a complex between benzoic acid and caffeine was discussed. The present study extends the investigation to benzoate ion and suggests existence of more than one complex between caffeine and benzoic acid.

The phenomenon of molecular association, responsible for the existence of these complexes, is important not only because of its possible influence on the stability and appearance of pharmaceutical preparations, but also because it often determines the pharmacological efficacy of drugs and even the biochemical mechanism by which they operate. The tendency of caffeine to associate with certain pharmaceuticals is similar to that exhibited by body fluids and tissues in binding drugs and appears to be somewhat analogous to the enzyme-substrate or holoenzyme-coenzyme reactions.

Because of the pharmaceutical and biological importance of molecular complexes, studies were undertaken to obtain physical-chemical data and to clarify the structure of some intricate complexes.

Most studies on the xanthines have been concerned only indirectly with the formation of complexes. Because of the limited solubilities of the free xanthines in water, the emphasis in the literature has been on patents for solubilizing agents for these substances. In most cases, solubilization has been achieved by formation of soluble complexes. The types of systems employed are summarized in Table I.

Although suggestions have been made in nonpatent literature that the increased solubility of the xanthines in the presence of other compounds was caused by the formation of soluble complexes, no extensive attempt was made to show mathematically the magnitude of the equilibrium involved. In 1910, Pellini (16, 17) reported the existence of a soluble complex be-

TABLE I.—A SUMMARY OF SUBSTANCES USED TO SOLUBILIZE XANTHINES

Xanthine	Solubilizing Agent	Reference
Caffeine	Acid sodium metaphosphate	(2)
Theophylline	Piperazine, ethylene diamine, hexamethyl diamine, lysidine	(3)
Theophylline	Sodium salicylate	(4)
	Sodium benzoate	(4)
	Potassium benzoate	(4)
N-Acetyltheobromine	Sodium salicylate	(4)
	Lithium salicylate	(4)
	Ammonium salicylate	(4)
	Potassium salicylate	(4)
Theobromine	Sodium salicylate	(4)
Theobromine or theophylline	Calcium or strontium hydroxide plus salicylic acid	(5)
Theophylline	Diethanolamine	(6)
Caffeine	Mandelic acid	(7)
Theophylline, theobromine, and caffeine	Adenosine	(8)
Theophylline	Primary and secondary aliphatic monoamines	(9)
Theophylline	Piperazine	(10)
	Ethylenediamine	(10)
Theophylline	Mono-, di-, and tri-isopropanolamine	(11)
Theophylline	Mono-aminopolyhydric alcohols	(12)
Theobromine or theophylline	Sodium salicylate	(13)
	Strontium salicylate	(13)
	Calcium salicylate	(13)
Caffeine, theobromine, or theophylline	N-Methylbenzamide	(14)
	N-N′-Dimethylbenzamide	(14)
	p-Toluylic dimethylamide	(14)
	α-, β-, and γ-Pyridine carboxylic diethylamide	(14)

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Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

† This paper is based in part on a thesis submitted to the Graduate School of the University of Wisconsin by D. A. Zuck in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Fellow of the American Foundation for Pharmaceutical Education. Present address: Eli Lilly and Co., Indianapolis, Ind.

tween caffeine and sodium benzoate, supporting his statement by cryosopic measurements. In 1924, Regenbogen and Schoorl (18) suggested that salicylic acid and caffeine formed a eutectic mixture when combined in molecular proportions, but in 1929 Schoorl considered this mixture a definite molecular compound (19). In 1921, Emery and Wright (20) made a comprehensive study of the solubility and distribution coefficient of caffeine in the presence of a number of complexing agents but did not analyze mathematically the several equilibria involved. In 1930, Labes (21) determined the equilibrium constant between salicylate ion and caffeine. Chambon (22) described a phase rule study of the caffeine-sodium benzoate-water system, but did not present quantitative data on the stability of the complexes postulated. Paul (23) reported that his diffusion rate studies on xanthines and their solubilizing agents did not indicate the formation of complexes.

In 1948, Neish (15) found that caffeine increased the solubility of a number of aromatic amines, including naphthyl amine, sulfapyridine, sulfathiazole, and certain dyes. This effect may be due to some type of complex formation.

In the present investigation, the emphasis has been placed on the quantitative evaluation of the stabilities of the complexes, the energy changes involved in their formation, and the influence of structure on these quantities.

BENZOIC ACID AND CAFFEINE

Data from previous studies (1) of the interaction of benzoic acid and caffeine at 0° have been included to permit fuller mathematical analysis, and the investigation has been extended to other temperatures. An attempt has been made to explain some of the observed deviations in the equilibrium constant by postulating an additional reaction.

Determination of the Degree of Interaction between Caffeine and Benzoic Acid at 15° and at 30°.—The experimental procedure for the determination of the partition coefficient of benzoic acid, the concentration of free benzoic acid in solution, and the benzoic acid content in the form of a complex at 15° and at 30° was essentially the same as that employed for the studies at 0°.

Results.—The partition coefficients of benzoic acid between water and Skellysolve-C at 0°, 15°, and 30° are represented in Fig. 1, and have been used to determine the concentration of free benzoic acid and the amount of benzoic acid complex. Data are given in Tables II–IV with varying experimental conditions. The equilibrium constants were calcu-

TABLE II.—DATA SHOWING INTERACTION BETWEEN BENZOIC ACID AND CAFFEINE AT 0°

Original Concn. Caffeine, $\times 10^{-3}$ M	Total Acid Found, $\times 10^{-3}$ M	Total Nondissoc. Acid, $\times 10^{-3}$ M	Free Benzoic Acid,[a] $\times 10^{-3}$ M	Total Acid Bound as Complex,[b] $\times 10^{-3}$ M	Calcd. Total Acid Bound as Complex,[c] $\times 10^{-3}$, M	K_c[d]
For 1.0 Gm. of Benzoic Acid Added per 50 Ml. of Water						
10.36	16.23	15.25	11.94	3.31	3.31	39.4
10.30	16.23	15.25	11.92	3.33	3.26	40.1
15.41	17.92	16.89	11.94	4.95	4.92	39.6
20.60	19.56	18.48	11.94	6.54	6.59	38.9
26.91	21.53	20.40	11.94	8.46	8.62	38.4
30.90	22.85	21.69	11.93	9.76	9.86	40.6
For 0.25 Gm. of Benzoic Acid Added per 50 Ml. of Water						
5.16	14.13	13.22	11.62	1.60	1.58	38.5
10.30	15.48	14.52	11.33	3.19	3.09	39.6
15.52	16.69	15.70	11.08	4.62	4.60	38.2
20.64	17.87	16.84	10.82	6.02	6.03	38.1
25.85	18.99	17.93	10.57	7.36	7.28	39.8
31.00	19.98	18.89	10.32	8.57	8.68	37.1
36.04	20.86	19.74	10.06	9.68	9.87	36.5
41.27	21.89	20.75	9.85	10.91	11.97	36.5
46.38	22.83	21.66	9.65	12.01	12.21	36.2
For 0.15 Gm. of Benzoic Acid Added per 50 Ml. of Water						
15.50	12.29	11.44	7.98	3.46	3.31	35.9
20.65	13.05	12.17	7.71	4.47	4.39	35.8
25.84	13.76	12.86	7.48	5.38	5.35	35.1
30.92	14.34	13.42	7.26	6.16	6.22	35.7
36.05	14.87	13.93	6.99	6.94	7.00	34.2
41.37	15.46	14.51	6.76	7.74	7.78	34.2
46.59	15.94	14.97	6.57	8.41	8.54	33.6

[a] Calculated from acid concentration in the organic layer.
[b] The difference between the third and fourth column. Some of the calculations are not in exact agreement since the numbers were rounded off after calculation.
[c] This column represents the amount of complexed acid, assuming the dual complex theory and the K'_c and K''_c values given in the text.
[d] These equilibrium constants were calculated assuming the formation of only one complex form described by Eq. 2.

TABLE III.—DATA SHOWING INTERACTION BETWEEN BENZOIC ACID AND CAFFEINE AT 15°

Original Concn. of Caffeine, $\times 10^{-3} M$	Total Acid Found, $\times 10^{-3} M$	Total Nondissoc. Acid, $\times 10^{-3} M$	Free Benzoic Acid,[a] $\times 10^{-3} M$	Total Acid Bound as Complex,[a] $\times 10^{-3} M$	Calculated Total Acid Bound as Complex,[a] $\times 10^{-3} M$	K_c[a]
For 1.0 Gm. of Benzoic Acid Added per 50 Ml. of Water						
41.30	35.00	33.55	18.57	14.98	15.19	30.6
36.52	33.32	31.90	18.61	13.29	13.48	30.8
31.06	31.36	29.98	18.62	11.36	11.44	31.0
25.92	29.50	28.16	18.64	9.52	9.56	31.2
20.76	27.57	26.27	18.53	7.76	7.57	32.2
15.39	25.48	24.25	18.51	5.73	5.62	32.1
10.23	23.65	22.46	18.64	3.82	3.76	31.9
For 0.75 Gm. of Benzoic Acid Added per 50 Ml. of Water						
45.15	36.89	35.40	18.56	16.83	16.52	30.6
38.78	34.24	32.80	18.62	14.18	14.31	30.9
38.78	34.06	32.63	18.59	14.04	14.30	30.5
30.94	31.21	29.84	18.62	11.22	11.45	30.6
20.80	27.54	26.25	18.65	7.60	7.68	30.9
15.45	25.47	24.24	18.60	5.64	5.70	30.9
10.30	23.58	22.39	18.61	3.78	3.78	31.2
For 0.50 Gm. of Benzoic Acid per 50 Ml. of Water						
44.30	33.29	31.87	16.96	14.91	15.08	31.3
38.66	31.84	30.46	17.20	13.27	13.28	30.4
30.90	29.74	28.40	17.57	10.84	10.83	30.8
23.10	27.72	26.43	17.89	8.55	8.14	32.9
17.25	25.94	24.70	18.22	6.48	6.18	33.0
10.40	23.78	22.59	18.62	3.96	3.91	31.6
For 0.25 Gm. of Benzoic Acid Added per 50 Ml. of Water						
44.38	21.68	20.54	10.65	9.89	9.96	26.9
38.66	20.86	19.74	10.91	8.83	8.88	27.2
31.06	19.56	18.49	11.20	7.29	7.31	27.4
23.86	18.38	17.33	11.53	5.81	5.75	27.9
17.66	17.19	16.18	11.85	4.33	4.38	27.4
10.87	15.89	14.92	12.17	2.77	2.76	27.9

[a] See footnotes to Table II.

lated by assuming that only one reaction took place according to Eq. 1:

$$\text{Caffeine} + \text{HBz} \rightleftarrows \text{Caffeine}-\text{HBz} \qquad (1)$$

The equilibrium constant for this reaction (Eq. 2) is then:

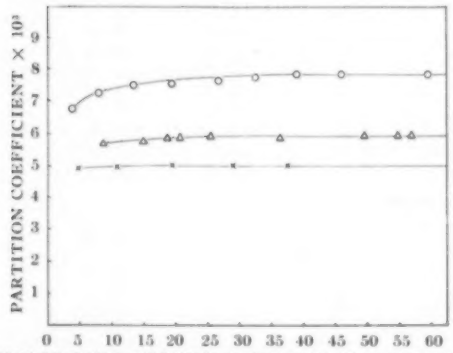

Fig. 1.—Partition coefficient of benzoic acid between Skellysolve-C and water. x——x, 0°; Δ——Δ, 15°; o——o, 30°.

$$K_c = \frac{[\text{Caffeine}-\text{HBz}]}{[\text{Caffeine}]\,[\text{HBz}]} \qquad (2)$$

The equilibrium constants thus determined are given in the tables to show that they are independent of the caffeine concentration at any given concentration of benzoic acid.

Some deviation in the equilibrium constant value is apparent, however, when the concentration of benzoic acid is varied. In an attempt to explain this, it was assumed that an additional reaction, (Eq. 3), took place:

$$\text{HBz} + (\text{Caffeine}-\text{HBz}) \rightleftarrows (\text{Caffeine}-2\text{HBz}) \qquad (3)$$

The equilibrium constant for this second reaction can be written (Eq. 4):

$$K''_c = \frac{[\text{Caffeine}-2\text{HBz}]}{[\text{Caffeine}-\text{HBz}]\,[\text{HBz}]} \qquad (4)$$

By a trial and error approach it was possible to solve for values of K'_c[1] and K''_c which uniquely

[1] Henceforth the symbol $K'c$ will represent the equilibrium constant,

$$K'_c = \frac{[\text{Caffeine}-\text{HBz}]}{[\text{Caffeine}]\,[\text{HBz}]}$$

where reaction 3 is also assumed to be taking place. $K''c$ will have the meaning defined by Eq. 4. The symbol K_c will retain the earlier meaning of reaction 1 but without allowing for the existence of the second reaction. In the latter case it is assumed in calculating the constant that all of the complexed acid is in the form of the simpler complex.

TABLE IV.—DATA SHOWING INTERACTION BETWEEN BENZOIC ACID AND CAFFEINE AT 30°

Original Concn. of Caffeine, $\times 10^{-3}$ M	Total Acid Found, $\times 10^{-3}$ M	Total Nondissoc. Acid, $\times 10^{-3}$ M	Free Benzoic Acid,[a] $\times 10^{-3}$ M	Total Acid Bound as Complex,[a] $\times 10^{-3}$ M	Calculated Total Acid Bound as Complex,[a] $\times 10^{-3}$ M	K_c[a]
For 1.0 Gm. of Benzoic Acid Added per 50 Ml. of Water						
30.84	45.38	43.73	30.03	13.70	13.56	26.6
25.92	43.50	41.88	30.18	11.70	11.37	27.3
20.70	41.14	39.56	30.21	9.36	9.10	27.3
20.70	41.02	39.45	30.16	9.29	9.14	27.0
15.73	38.54	37.02	30.07	6.95	6.92	26.3
10.65	36.51	35.02	30.17	4.86	4.68	27.8
For 0.75 Gm. of Benzoic Acid Added per 50 Ml. of Water						
44.72	42.88	41.27	24.75	16.52	16.92	23.7
36.04	40.37	38.81	25.20	13.60	13.80	24.1
30.90	38.64	37.11	25.42	11.69	11.93	24.0
25.74	37.06	35.56	25.66	9.90	10.02	24.4
20.62	35.45	33.99	25.92	8.07	8.06	24.8
15.45	33.77	32.34	26.22	6.12	6.08	25.0
10.30	32.13	30.74	26.55	4.19	4.07	25.8
For 0.50 Gm. of Benzoic Acid Added per 50 Ml. of Water						
41.32	33.14	31.72	19.47	12.26	12.74	21.7
36.30	32.07	30.68	19.67	11.01	11.25	22.1
36.82	32.01	30.62	19.67	10.96	11.56	21.6
31.42	30.70	29.35	19.84	9.51	9.82	21.9
25.98	29.50	28.16	20.12	8.04	8.18	22.3
25.78	29.42	28.09	20.12	7.97	8.25	22.2
20.86	28.16	26.86	20.36	6.50	6.77	22.2
15.47	26.80	25.53	20.62	4.92	4.99	22.6
10.32	25.33	24.10	20.84	3.26	3.37	22.2
For 0.25 Gm. of Benzoic Acid Added per 50 Ml. of Water						
41.28	21.41	20.28	12.12	8.16	8.28	20.3
35.94	20.67	19.56	12.27	7.29	7.30	20.7
32.14	20.14	19.05	12.45	6.60	6.60	20.8
25.94	19.34	18.27	12.76	5.51	5.42	21.1
25.96	19.29	18.22	12.76	5.46	5.44	20.9
20.58	18.49	17.44	13.01	4.44	4.40	21.1
15.75	17.82	16.79	13.24	3.55	3.46	22.0
15.51	17.66	16.64	13.23	3.41	3.38	21.3

[a] See footnotes to Table II.

satisfied the experimental data over the entire range of benzoic acid and caffeine concentrations studied. These calculated stability constant values are given below:

Temperature, ° C.	K'_c	K''_c
0	29 ± 1	12 ± 1
15	23 ± 1	7.5 ± 0.5
30	18 ± 1	5.5 ± 0.5

The ± values indicate the estimated probable errors in the calculation. The assumption of the second reaction results in a sizable reduction in the calculated equilibrium constant for the first reaction, since the presence of the higher complex in solution leads to a corresponding reduction in the amount of the complexed acid attributable to the simpler complex.

The expected total amounts of benzoic acid which would be bound in the form of the two complexes under the experimental conditions have been calculated on the basis of the two sets of equilibrium constants given, and are compared with determined values (Tables II–IV). It is evident from the comparative values of the equilibrium constants that the assumption of the two reactions closely simulates the actual situation, and that the calculated total bound acid concentration is equal to the determined concentration within little more than experimental error in nearly all cases.

TABLE V.—SUMMARY OF EQUILIBRIUM CONSTANTS AND THERMODYNAMIC VALUES OF THE BENZOIC ACID-CAFFEINE INTERACTION

K'_c	Temp., ° C.	$F°$, Cal./Mole	H, Cal./mole	S, Cal./Deg./Mole
29	0	−1,850	...	−4
23	15	−1,800	3,000	−4
18	30	−1,750	...	−4
K''_c	Temp., ° C.	$F°$, Cal./Mole	H, Cal./Mole	S, Cal./Deg./Mole
12	0	−1,350	....	−10
7.5	15	−1,150	4,000	−10
5.5	30	−1,050	...	−10

Thermodynamic Calculations.—Since the K'_c and K''_c values at several temperatures were available, a number of thermodynamic constants for the two reactions could be determined and were calculated as shown in Eqs. 5 and 6. Results are summarized in Table V.

(*a*) *Free energy change:*

$$-\Delta F^\circ = RT \ln K_c \qquad (5)$$

(*b*) *Heat of formation or reaction:*

$$\ln K_c = \frac{\Delta H}{RT} + C \qquad (6)$$

In Fig. 2 the values of log K_c are plotted against $1/T$ and it is evident that a straight line is produced, as expected from Eq. 6. The heat of formation is calculated from the slope of the line (Fig. 2) as follows (Eq. 7):

$$\Delta H = -\text{slope} \times 2.303R \qquad (7)$$

(*c*) *Entropy change:* The entropy change was calculated using Eq. 8:

$$\Delta S = \frac{\Delta H - \Delta F}{T} \qquad (8)$$

since the heat of formation and free energy change were known.

Discussion.—From present experimental evidence it appears that the over-all behavior of the caffeine-benzoic acid system can be best rationalized on the basis of the dual complex theory. At each of the three temperatures employed, the equilibrium properties of the system adhere rather closely to those predicted by the two equilibrium constants, K'_c and K''_c, through wide variations in the concentrations of the two components. Moreover, the thermodynamic constants themselves lend some support to this hypothesis. The formation of the first complex is exothermic to the extent of roughly 3,000 cal./mole, corresponding approximately to the amount of energy equivalent to the formation of a single hydrogen bond. The reaction of the bimolecular complex with another molecule of benzoic acid appears to yield more energy than the first complex but of the same order of magnitude.[2] The third fact in favor of the theory is that many compounds form trimolecular complexes with caffeine, which will be shown later.

The direction and the magnitude of the entropy changes for the two equilibria conform to expectations. Formation of complexes from uncharged components usually results in an increase in the order of the systems involved and a subsequent decrease in entropy. The higher entropy change on formation of the second complex suggests a highly-ordered triangular type of structure for this molecular compound.

BENZOATE ION AND CAFFEINE

Although suggestions have been made by previous workers (17, 21, 23, 24) that the increased solubility of caffeine in the presence of sodium benzoate was due to formation of a soluble complex between the two compounds, no quantitative study of this interaction has been previously undertaken.

Two different methods were used in the study of the caffeine-benzoate ion interaction. The first method involved an organic and an aqueous phase and was somewhat analogous to the method used in the caffeine-benzoic acid interaction. Because of unsatisfactory results, a second method based on the changing solubility of caffeine was employed. Although this technique was very simple experimentally, it suffered from the fact that measurements could be made at only one concentration of caffeine corresponding to the activity of the solid.

Determination of the Degree of Interaction between Caffeine and Sodium Benzoate.—Sodium benzoate solutions were prepared in the following concentrations: 0.025, 0.050, 0.100, 0.200, 0.300, 0.400, and 0.500 *N*. An excess of caffeine was added to 50 ml. of distilled water and to 50 ml. of each of the sodium benzoate solutions in glass-stoppered bottles which were then placed in a constant temperature water bath at 30, 15, and 0°. After continuous shaking for twenty-four hours, the bottles were suspended in the water bath to allow the solids to settle. The concentration of caffeine was determined spectrophotometrically. Sodium benzoate solution of the proper dilution was used as a blank in each case.

Results.—Results obtained at 30, 15, and 0° are tabulated in Tables VI, VII, and VIII. Figure 2 shows the effect of sodium benzoate on the solubility of caffeine at the three different temperatures. The equilibrium constants were calculated on the basis of Eq. 9 below.

TABLE VI.—INTERACTION OF SODIUM BENZOATE AND CAFFEINE AT 30°

Concn. of Na Benzoate, Moles/L.	Satd. Caffeine Concn., $\times 10^2$ *M*	Complex Concn., $\times 10^2$ *M*[a]	Equilibrium Constant
0.000	13.33	...	..
0.025	14.78	1.453	10.4
0.050	16.49	3.156	12.8
0.100	19.39	6.063	11.6
0.200	27.69	13.46	15.4
0.200	26.66	13.33	15.0
0.300	32.39	19.06	13.1
0.300	32.50	19.17	13.3
0.400	37.17	23.84	11.1
0.500	44.85	31.52	12.8
0.500	46.30	32.97	14.5
		Av.	13.0

[a] Calculated by assuming one molecule of caffeine present per molecule of complex. It is determined by subtracting caffeine solubility in presence and absence of benzoate.

TABLE VII.—INTERACTION BETWEEN SODIUM BENZOATE AND CAFFEINE AT 15°

Concn. of Na Benzoate, Moles/L.	Satd. Caffeine Concn., $\times 10^2$ *M*	Complex Concn., $\times 10^2$ *M*[a]	Equilibrium Constant
0.000	6.603	....	..
0.100	11.254	4.651	13.2
0.200	15.248	8.645	11.5
0.300	21.834	15.231	15.6
0.400	26.038	19.435	14.3
0.500	33.742	27.139	18.0
0.000	6.624	....	..
0.200	17.027	10.403	16.4
0.300	22.258	15.634	16.4
0.500	34.366	27.742	18.8
		Av.	15.5

[a] See footnote to Table VI.

[2] Actually these reactions give off much more than the measured amount of energy, since in all probability they are accompanied by endothermic dehydration reactions.

TABLE VIII.—INTERACTION BETWEEN SODIUM BENZOATE AND CAFFEINE AT 0°

Concn. of Na Benzoate, Moles/L.	Satd. Caffeine Concn., $\times 10^2$ M	Complex Concn., $\times 10^2$ M[a]	Equilibrium Constant
0.000	3.887	....	..
0.200	12.584	8.696	19.8
0.300	18.563	14.676	24.6
0.300	18.023	14.136	22.9
0.500	27.742	23.855	23.5
		Av.	22.7

[a] See footnote to Table VI.

Thermodynamic Calculations.—The free energy change, the heat of reaction, and the entropy change were calculated as for benzoic acid. The equilibrium constant values were determined from the graph (Fig. 3). Results are summarized in Table IX.

Discussion.—The results from the investigation of the interaction of caffeine and the benzoate ion do not support the possibility of two reactions taking place such as are represented by Eqs. 9 and 10:

$$\text{Caffeine} + \text{Benzoate}^- \rightleftarrows [\text{Caffeine-Benzoate}^-] \quad (9)$$

$$\text{Caffeine-Benzoate}^- + \text{Benzoate}^- \rightleftarrows [\text{Caffeine-(Benzoate)}_2]^- \quad (10)$$

TABLE IX.—SUMMARY OF RESULTS FOR THE CAFFEINE-BENZOATE INTERACTION

Temp., ° C.	K_c	Slope of Line Log K_c vs. $1/T$	$\Delta F°$, Cal./ Mole	ΔH, Cal./ Mole	ΔS, Cal./ Mole/ Deg.
0	23.1	...	−1,700	−3,300	−6
15	16.4	715	−1,600	−3,300	−6
30	12.8	...	−1,540	−3,300	−6

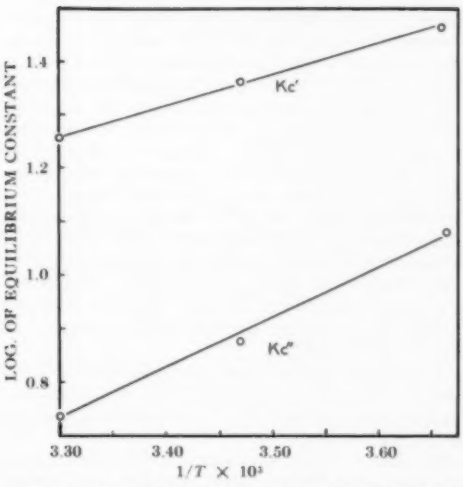

Fig. 2.—Log K_c' and log K_c'' plotted against the reciprocal of absolute temperature; the slopes of these lines are proportional to the respective heats of reaction.

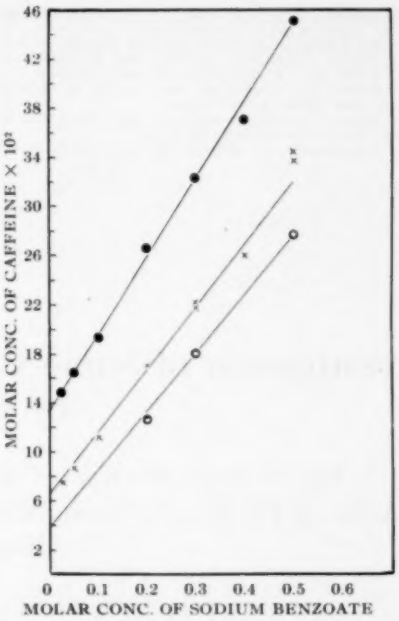

Fig. 3.—Solubility of caffeine in sodium benzoate solution. ●——●, at 30°; x——x, at 15°; o——o, at 0°.

Although the existence of complexes of the form represented by Eqs. 9 and 10 was suggested by Chambon (22), it is difficult to conceive that a second benzoate cation would associate with a negatively charged caffeine-benzoate complex because of the repulsion of like-charged bodies. The essentially straight line dependency of caffeine solubility on sodium benzoate concentration also indicates that the complex must be substantially monomolecular with respect to benzoate ion under the prevailing experimental conditions.

The results do not, however, preclude the possibility of a reaction of the type

$$\text{Benzoate}^- + 2\ \text{Caffeine} \rightleftarrows [\text{Benzoate-2 Caffeine}]^- \quad (11)$$

as being the principal reaction, since the dependency of the complexing reaction on caffeine was not studied.

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Investigation of Some Complexes Formed in Solution by Caffeine*,†

III. Interactions Between Caffeine and Aspirin, *p*-Hydroxybenzoic Acid, *m*-Hydroxybenzoic Acid, Salicylic Acid, Salicylate Ion, and Butyl Paraben

By TAKERU HIGUCHI and D. A. ZUCK‡

The equilibrium reactions leading to complex formation between caffeine and six pharmaceuticals have been studied. The stability constants at several temperatures, the heats of reaction, and the entropy changes have been approximated. It is shown that complex formation may lead to either a decrease or an increase in the solubilities of the reactants. The influence of structure on equilibrium is also discussed.

IN THE preceding papers of this series (1, 2) the nature of interaction of caffeine with benzoic acid and benzoate ion was discussed. The present investigation is concerned with a similar study of complex formation between the xanthine and a number of other pharmaceutical compounds which were chosen to obtain some insight as to the structural factors favoring complex formation. In this study data are presented for aspirin, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, salicylate ion, and butyl paraben.

ASPIRIN

Although the use of aspirin and caffeine together in pharmaceutical preparations is quite extensive, no attempt seems to have been made to determine whether an interaction takes place between caffeine and aspirin in aqueous solution. Such a reaction may be expected, since similar aromatic acids have been found to form complexes with caffeine.

Reagents.—Recrystallized aspirin U. S. P., m. p. 133–135°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.001 *N* sulfuric acid; 0.0200 *N* alcoholic potassium hydroxide; α-naphtholbenzein indicator.

Determination of the Degree of Interaction between Aspirin and Caffeine.—Excess quantities (1.0 Gm. at 30° and 0.70 Gm. at 15°) of aspirin, accurately weighed, were placed in 125-ml. glass-stoppered bottles together with varying but accurately weighed amounts of caffeine (0.2 to 1.5 Gm.), and the bottles were brought to the temperature of the experiment. A quantity of 100 ml. of dilute sulfuric acid solution, at the experiment temperature, was added to each bottle. The bottles were placed in a mechanical shaker in a constant temperature water bath and were shaken for three hours at 30° and for six hours at 15°. Aliquot portions were then removed and titrated with 0.0200 *N* alcoholic potassium hydroxide solution to determine the total concentration of aspirin in solution; α-naphtholbenzein was the indicator used. All titrations were carried out under an atmosphere of nitrogen. A blank was also run.

The dilute sulfuric acid solution (0.001 *N*) was used as the solvent to suppress any dissociation of aspirin ($K_a = 3.33 \times 10^{-4}$ at 25°) and to decrease its hydrolytic rate.

Results.—The assumption of the following reaction appears to be in accord with the experimental data:

$$\text{Aspirin} + \text{Caffeine} \rightleftarrows [\text{Aspirin-Caffeine}] \quad (1)$$

* Received August 22, 1952, from the School of Pharmacy, University of Wisconsin, Madison.
Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.
† This paper is based in part on a thesis submitted to the Graduate School of the University of Wisconsin by D. A. Zuck in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
‡ Fellow of the American Foundation for Pharmaceutical Education. Present address: Eli Lilly and Co., Indianapolis, Ind.

The equilibrium constant can be written as:

$$K_c = \frac{[\text{Aspirin-Caffeine}]}{[\text{Aspirin}]\,[\text{Caffeine}]} \qquad (2)$$

The solubility of aspirin in the absence of caffeine corresponded to the concentration of free aspirin molecules in solution. The difference between the total aspirin concentration and the free aspirin concentration was thus the concentration of aspirin complex. The difference between the original caffeine concentration and the complex concentration was the concentration of free caffeine in solution. The concentration of total aspirin was corrected for hydrolysis in each case. By substituting these values in Eq. 2, the equilibrium constant was determined over a wide range of caffeine concentrations. Results obtained are tabulated in Tables I and II. Figure 1 represents the solubilizing action of caffeine on aspirin; the broken line shows ideal behavior, from which equilibrium constants at 15° and 30° were obtained for the determination of thermodynamic values.

It must be kept in mind that the aspirin-caffeine complex may contain more than one aspirin molecule per complex since the experimental data were obtained at constant aspirin activity. This seems unlikely, however, in view of the fact that the aspirin concentration was rather low in all of the systems studied.

Because of the gradual decrease in the equilibrium constant values with increase in caffeine concentration, these values were derived from the straight line representing the ideal behavior found in dilute solutions. This limiting value can also be determined by plotting the equilibrium constant values obtained

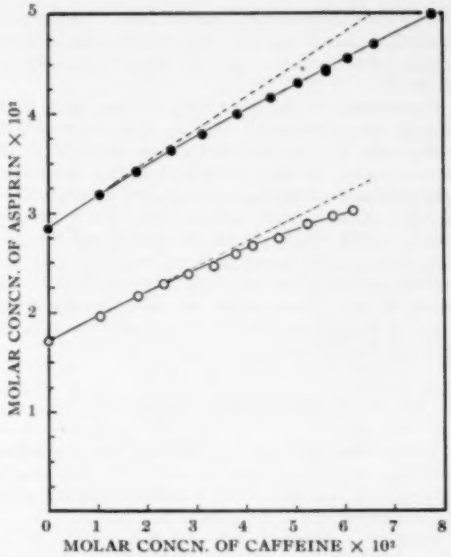

Fig. 1.—Solubilizing action of caffeine on aspirin. ●——● at 30°; o——o at 15°. The near-linear relationship indicates that the complex formed is monomolecular with respect to caffeine.

TABLE I.—INTERACTION OF ASPIRIN AND CAFFEINE IN AQUEOUS SOLUTION AT 15°

Orig. Caffeine Concn., × 10³ *M*	Satd. Total Aspirin Concn., × 10³ *M*	Aspirin Concn. as Complex, × 10³ *M*	Free Caffeine Concn., × 10³ *M*	Equilibrium Constant[a]
0.0000	1.7159	....	....	...
1.0469	1.9704	0.2545	0.7924	18.71
1.8075	2.1709	0.4550	1.3525	19.60
2.3281	2.2828	0.5669	1.7612	18.76
2.8364	2.3926	0.6767	2.1597	18.26
3.3575	2.4775	0.7616	2.5959	17.10
3.7953	2.5835	0.8676	2.9277	17.27
4.1279	2.6838	0.9679	3.1600	17.85
4.6758	2.7532	1.0373	3.6385	16.61
5.2336	2.8785	1.1626	4.0710	16.64
5.7572	2.9614	1.2455	4.5117	16.08
6.1687	3.0270	1.3111	4.8576	15.73

[a] Assuming Eq. 1.

TABLE II.—INTERACTION OF ASPIRIN AND CAFFEINE IN AQUEOUS SOLUTION AT 30°

Orig. Caffeine Concn., × 10³ *M*	Satd. Total Aspirin Concn., × 10³ *M*	Aspirin Concn. as Complex, × 10³ *M*	Free Caffeine Concn., × 10³ *M*	Equilibrium Constant[a]
0.0000	2.8512	....	....	...
1.0598	3.1856	0.3344	0.7254	16.17
1.7905	3.4207	0.5695	1.2210	16.36
2.5001	3.6290	0.7778	1.7223	15.84
3.1098	3.7857	0.9345	2.1753	15.07
3.8400	4.0016	1.1504	2.6896	15.00
4.4930	4.1564	1.3052	3.1878	14.36
5.0494	4.3150	1.4638	3.5856	14.32
5.5924	4.4469	1.5957	3.9967	14.00
6.0492	4.5654	1.7142	4.3350	13.87
6.5750	4.6953	1.8441	4.7309	13.67
7.7579	4.9992	2.1480	5.6099	13.43

[a] Assuming Eq. 1.

against the caffeine concentration used. All thermodynamic values were then calculated in the manner outlined for benzoic acid and are summarized in Table III.

Discussion.—It is interesting to note that, with aspirin, the addition on the ring of an ester substituent *ortho* to the carboxyl group resulted in no enhancement of the complex-forming tendency, although such groupings are generally considered to be quite polar. With salicylic acid and with salicylate, much more stable complexes are formed. The considerably lower heat of reaction and the slightly positive entropy change indicate that the degree of interaction among the components of the complex is considerably less than with the benzoic acid-caffeine complexes.

SALICYLIC ACID

No previous quantitative study of the interaction between caffeine and salicylic acid has been made, although the reaction between caffeine and salicylate ion was investigated by Labes (3).

Reagents.—Recrystallized salicylic acid U. S. P., m. p. 159°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.003 *N* sulfuric acid solution; α-naphtholbenzein indicator; 0.0200 *N* alcoholic potassium hydroxide.

Determination of the Degree of Interaction between Salicylic Acid and Caffeine.—Excess quantities of salicylic acid (0.70 Gm.) were weighed into 125-ml. glass-stoppered bottles. Varying amounts of caffeine (0.1 to 2.0 Gm.), accurately weighed, were added, and the mixture was heated to the reaction temperature. Dilute sulfuric acid solution (100 ml.), also at reaction temperature, was added to suppress any dissociation of salicylic acid ($K_a = 1.06 \times 10^{-3}$). The bottles were shaken continuously for four hours at 30° and for eight hours at 15°; aliquot portions were then titrated for total acid concentration in solution by the procedure used for aspirin.

Results.—The results are tabulated in Table IV. The molar concentration of the acid in solution has been plotted (Fig. 2) against the molar concentration of caffeine added.

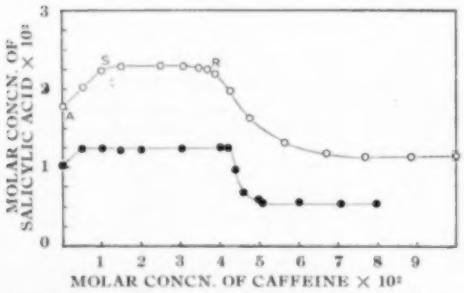

Fig. 2.—Solubility of salicylic acid as function of moles of caffeine present per liter of water. o——o at 30°; ●——● at 15°. The amount of caffeine shown is actually the amount added per liter of solvent. The system contained a total of 0.0506 mole/L. of salicylic acid in and out of solution.

TABLE III.—SUMMARY OF RESULTS FOR THE CAFFEINE-ASPIRIN INTERACTION

Temp., °C.	K_c	Slope of Line Log K_c vs. $1/T$	ΔH, Cal./Mole	ΔF, Cal./Mole	ΔS, Cal./Deg./Mole
15	19.8	335	−1500	−1700	0.6
30	17.4	335	−1500	−1700	0.6

TABLE IV.—SALICYLIC ACID AND CAFFEINE IN AQUEOUS SOLUTION

At 30°		At 15°	
Orig. Caffeine Concn., × 10^2 *M*	Total Satd. Concn. Salicylic Acid, × 10^2 *M*	Orig. Caffeine Concn., × 10^2 *M*	Total Satd. Concn. Salicylic Acid, × 10^2 *M*
0.000	1.796	0.000	1.028
0.512	2.024	0.499	1.227
0.996	2.238	1.014	1.227
1.450	2.290	1.483	1.208
2.498	2.286	2.009	1.217
3.002	2.280	3.038	1.242
3.458	2.268	3.991	1.261
3.680	2.253	4.022	1.251
3.888	2.192	4.205	1.251
4.262	1.985	4.389	0.975
5.631	1.324	4.591	0.684
6.702	1.189	4.998	0.597
7.691	1.137	5.075	0.558
8.856	1.142	5.997	0.563
9.993	1.153	7.070	0.548
...	...	7.937	0.548

Discussion.—It is evident (Table IV and Fig. 2) that the over-all reaction is relatively complex. At low caffeine concentration, a straight line relationship is obtained between the concentrations of acid and caffeine in solution. The slope of this portion (*A*–*S*) gives an equilibrium constant of 44 at 30°, assuming that the interaction between caffeine and salicylic acid takes the form:

$$\text{Caffeine} + \text{Salicylic acid} \rightleftarrows \text{Caffeine-Salicylic Acid} \quad (3)$$

It is believed that *S* represents the saturation point of the solution with respect to this complex, and that on further addition of caffeine, the complex (Eq. 3) continues to form until all the salicylic acid added, other than the free salicylic acid (*A*), associates with caffeine to form mainly an insoluble complex having the ratio of 1:1 with respect to caffeine and salicylic acid. At point R, the system is no longer saturated with respect to the acid, since all of the excess solid acid has been converted to the complex. Further addition of caffeine results in the depletion of acid in solution. However, it is not known whether the additional complex formed has a higher ratio with respect to caffeine. Formation of another complex is indicated by the fact that the limiting amount of acidic material in solution appears to be greater than the solubility of the initial complex.

Because of the difficulty in determining the over-all equilibrium constant at several temperatures, no thermodynamic calculations were made.

When the initial caffeine concentration was greater than 1.0×10^{-2} *M*, a white precipitate

formed which was removed by filtration and recrystallized three times from hot water; m. p. 139.5 to 140.5°. In 1929, Schoorl (4) prepared a double compound by dissolving equimolar quantities of caffeine and salicylic acid in hot solution. On cooling, a crystalline compound, m. p. 137°, formed.

The molecular weight of the precipitate was determined as 332.85 (calcd. 333.31). It appears that the complex caffeine-salicylic acid is stable but that other possible types of complexes such as (caffeine)$_2$-salicylic acid or (caffeine)$_2$-(salicylic acid)$_2$ readily dissociate to form caffeine-salicylic acid.

SALICYLATE ION

Because of the complexing tendency observed qualitatively for the system by earlier workers, a study of the extent of the interaction involved between caffeine and salicylate ion was made.

Reagents.—Recrystallized sodium salicylate U. S. P.; recrystallized caffeine U. S. P., m. p. 235–237°.

Determination of the Degree of Interaction between Salicylate Ion and Caffeine.—The experimental procedure was similar to that for benzoate ion. The shaking time was fifteen hours and the caffeine concentrations were determined spectrophotometrically.

As a check, aliquots of the solution were evaporated to determine caffeine concentration in solution. These results, indicated by crosses (X) in Fig. 3, show very close agreement with the spectrophotometric determinations.

Results.—Results obtained at 30° and at 15° are tabulated in Table V. The molar concentration of caffeine was plotted against the molar concentration of sodium salicylate (Fig. 3).

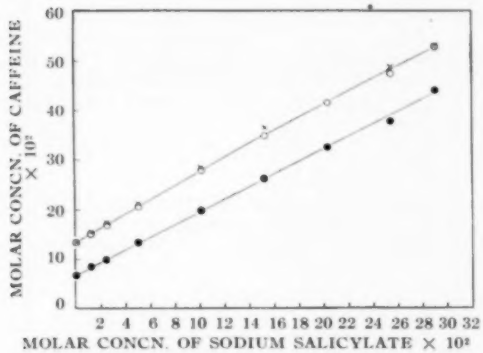

Fig. 3.—Solubility of caffeine in the presence of sodium salicylate. o——o at 30°; ●——● at 15°. The solubility curve is first order with respect to the salicylate but the indicated slope of greater than one in these plots is evidence of the formation of complexes containing more than one caffeine molecule per complex.

To determine whether there was any difference between the affinity of caffeine for salicylate ion and for salicylic acid, the following additional experiment was carried out.

TABLE V.—SODIUM SALICYLATE AND CAFFEINE AT 30° AND AT 15°

Concn. of Added Sodium Salicylate, × 10¹ *M*	Satd. Concn. of Caffeine at 30°, × 10¹ *M*	Satd. Concn. of Caffeine at 15°, × 10¹ *M*
0.000	1.343	0.671
0.128	1.499	0.837
0.255	1.682	0.984
0.510	2.045	1.312
1.020	2.782	1.960
1.530	3.457	2.604
2.040	4.132	3.229
2.550	4.734	3.758

A solution 0.01301 *M* with respect to salicylic acid and to sodium salicylate was prepared. Various known amounts of caffeine were added and the solution was shaken continuously at 30° for about three hours. The *p*H's of the solutions were determined; see Table VI.

TABLE VI

Concn. of Caffeine, × 10⁻¹ *M*	*p*H
0.0000	3.10
0.0242	3.10
0.0597	3.10
0.1076	3.10
0.2101	3.10
0.4082	3.10
0.7206	3.20
1.0299	3.20
1.3358	3.25
1.7452	3.32
2.0562	3.32[a]

[a] This sample was saturated with respect to caffeine.

From this table it can be seen that, over a fairly wide range of caffeine concentration, there is no change in the *p*H. Caffeine evidently has no greater affinity for salicylic acid than the salicylate ion or vice versa. The slight increase in the *p*H at higher caffeine concentration may be due to reasons other than complex formation.

p-HYDROXYBENZOIC ACID

The interaction between *p*-hydroxybenzoic acid and caffeine was chosen for study to gain information on the effect of spatial distribution of the polar functional groups, since this acid is isomeric with salicylic acid.

Reagents.—Recrystallized *p*-hydroxybenzoic acid, m. p. 214–215°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.005 *N* sulfuric acid; 0.0500 *N* alcoholic potassium hydroxide; 0.0500 *N* α-naphtholbenzein indicator.

Determination of the Degree of Interaction between *p*-Hydroxybenzoic Acid and Caffeine.—The experimental procedure was identical with that for aspirin except that 2.0 Gm. of *p*-hydroxybenzoic acid was used as an excess. A weakly acidic (0.005 *N*) sulfuric acid solution was employed as solvent to suppress any dissociation of the *p*-hydroxybenzoic acid (K_a = 2.5 × 10⁻⁵ at 0°). The bottles were shaken for four hours at 30° and for eight hours at 15°.

Results.—Data determined at 30° and at 15° are tabulated in Tables VII and VIII. The solubilizing effect of caffeine on the solubility of *p*-hydroxybenzoic acid is represented in Figs. 4 and 5. The molar concentration of the complex, at 30°, was the difference in solubility of *p*-hydroxybenzoic acid in the presence of caffeine and in the absence of caffeine.

TABLE VII.—*p*-HYDROXYBENZOIC ACID AND CAFFEINE IN AQUEOUS SOLUTION AT 30°

Orig. Caffeine Concn., $\times 10^2$ *M*	Satd. Total Acid Concn., $\times 10^2$ *M*	Acid Concn. as Complex, $\times 10^2$ *M*	Free Caffeine Concn., $\times 10^2$ *M*
0.000	5.421	...	...
0.512	5.931	0.510	0.002
0.998	6.411	0.990	0.008
1.457	6.851	1.430	0.027
1.457	6.871	1.450	0.007
2.518	7.921	2.501	0.018
3.530	8.902	3.481	0.049
4.048	9.422	4.001	0.047
4.048	9.437	4.016	0.032
4.761	10.147	4.726	0.035
5.621	10.897	5.476	0.199
6.682	11.772	6.351	0.330
7.675	12.723	7.301	0.374
8.798	13.573	8.152	0.646

Discussion.—Results obtained at 30°, (Fig. 4), indicate that for the initial quantity of caffeine added, an equimolar amount of *p*-hydroxybenzoic acid was solubilized. At higher caffeine concentrations, there is a gradual decrease from the straight line relationship, possibly due to caffeine associating with itself at higher caffeine concentrations or to formation of another complex with more than one molecule of caffeine per molecule of complex.

On the assumption that the interaction takes place according to Eq. 4,

TABLE VIII.—*p*-HYDROXYBENZOIC ACID AND CAFFEINE IN AQUEOUS SOLUTION AT 15°

Orig. Caffeine Concn., $\times 10^2$ *M*	Total Acid Concn., $\times 10^2$ *M*
0.000	2.624
0.256	2.794
0.507	2.983
0.551	3.031
1.004	3.372
1.506	3.776
1.976	4.035
2.477	4.482
3.050	4.705
3.491	4.724
3.514	4.535
3.620	4.161
3.767	3.895
3.932	3.822
4.045	3.759
4.270	3.684
4.521	3.684
5.021	3.681
6.037	3.681
6.717	3.686
7.941	3.672

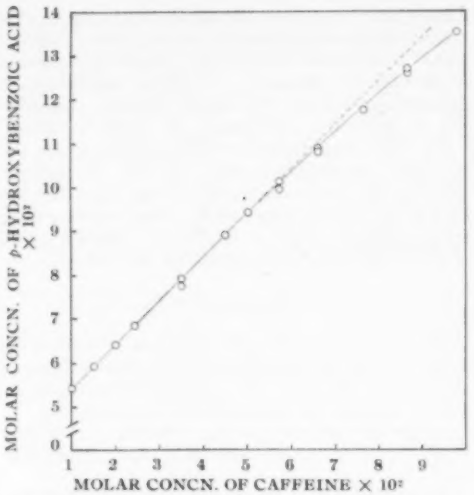

Fig. 4.—Solubilizing action of caffeine on *p*-hydroxybenzoic acid at 30°. The slope of the line is very nearly one, indicating formation of 1:1 complex with great stability.

$$\text{Caffeine} + \text{HA} \rightleftarrows \text{Caffeine}-\text{HA} \qquad (4)$$

the equilibrium constant can be written as

$$K_e = \frac{[\text{Caffeine}-\text{HA}]}{[\text{Caffeine}]\,[\text{HA}]} \qquad (5)$$

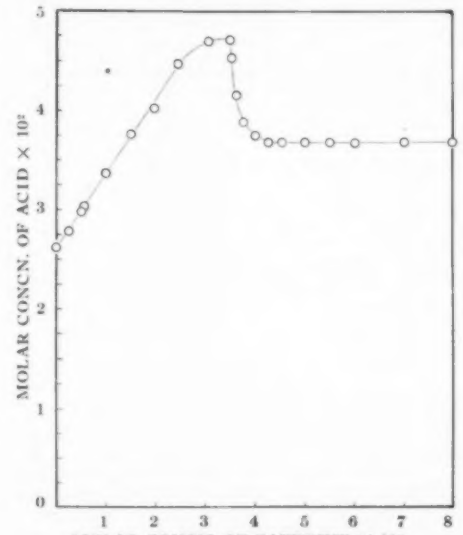

Fig. 5.—Influence of caffeine on the apparent solubility of *p*-hydroxybenzoic acid at 15°. The peak of the curve represents the system supersaturated with respect to the complex and saturated with respect to the free acid. The plateau to the right of the peak represents saturation with respect to both components. The initial straight line segment has a slope of one.

By using Eq. 5, a large equilibrium constant value would be obtained, since little free caffeine remains in solution. The equilibrium constant for this reaction cannot be precisely determined but is assumed to be at least greater than 100.

At 15°, the behavior of *p*-hydroxybenzoic acid was somewhat similar to that of salicylic acid. The concentration of the acid in solution (Fig. 5) increased rapidly with the initial addition of caffeine, followed by a sharp decrease which leveled off to a constant concentration independent of the amount of caffeine added.

A white flocculent precipitate, formed at 15°, was removed and recrystallized three times from hot water; m. p. 146–147°. The average molecular weight for a 1:1 acid-caffeine ratio was determined as 333.30 (calcd., 333.31). Apparently a complex is formed between *p*-hydroxybenzoic acid and caffeine which is only slightly soluble at 15° but is relatively soluble at 30°.

m-HYDROXYBENZOIC ACID

Since the behavior of *p*-hydroxybenzoic acid in the presence of caffeine was somewhat different from that of salicylic acid, the *m*-hydroxybenzoic acid-caffeine interaction was studied to correlate the position of the hydroxyl group in the ring with its over-all effect on the degree of interaction.

Reagents.—Recrystallized *m*-hydroxybenzoic acid, m. p. 201°; recrystallized caffeine U. S. P., m. p. 235–237°; 0.003 *N* sulfuric acid; 0.0500 *N* alcoholic potassium hydroxide; α-naphtholbenzein indicator.

Determination of the Degree of Interaction between Caffeine and *m*-Hydroxybenzoic Acid.—The experimental procedure was the same as that for aspirin. Since only a limited amount of the acid was available, only one series of determinations was made at 15°, using 1 Gm. of *m*-hydroxybenzoic acid and 50 ml. of 0.003 *N* sulfuric acid. The bottles were shaken for eight hours.

Results.—Data from this determination are tabulated in Table IX. The plot of acid concentration in solution against caffeine added is shown in Fig. 6.

TABLE IX.—M-HYDROXYBENZOIC ACID AND CAFFEINE IN AQUEOUS SOLUTION AT 15°

Orig. Caffeine Concn., × 10^2 *M*	Total Acid Concn., × 10^2 *M*
0.000	4.477
1.006	5.456
1.509	5.267
1.967	4.632
3.023	4.477
4.045	3.652
5.029	2.765

Discussion.—At low caffeine concentrations, the solubility of the acid was increased by addition of caffeine (Fig. 6). After a maximum was reached, there was a gradual decrease in the concentration of acid in solution. Further studies must be made to elucidate the mechanism of this rather complex behavior. It is difficult on the basis of the data presented to arrive at definite stoichiometric relationships.

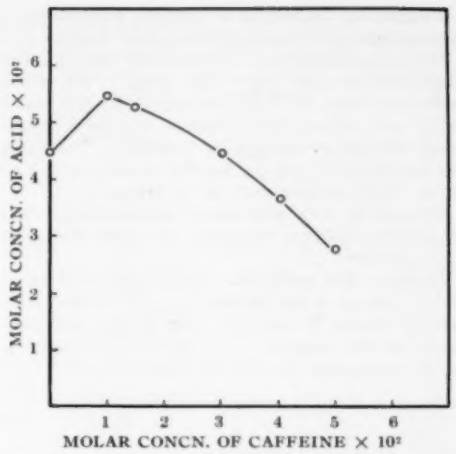

Fig. 6.—Influence of different amounts of caffeine on the apparent solubility of *m*-hydroxybenzoic acid.

The white flocculent precipitate which formed was filtered off and recrystallized three times from hot water; m. p. 159–160°. The average molecular weight for a 1:1 acid-caffeine ratio was determined as 332.4 (calcd. 333.31).

Although the formation of more than one type of complex is indicated, it appears that the complex which is stable to recrystallization is that in which the ratio is 1:1 with respect to caffeine and *m*-hydroxybenzoic acid.

BUTYL PARABEN

The study of the interaction between butyl paraben and caffeine was undertaken because of the similarity in structure between this compound and those previously discussed.

Reagents.—Purified butyl paraben, m. p. 72–73°; recrystallized caffeine U. S. P., m. p. 235–237°; 1% sodium carbonate solution.

Determination of the Degree of Interaction between Caffeine and Butyl Paraben.—Since the analysis was to be carried out spectrophotometrically, an ultraviolet absorption curve was prepared using a concentration of butyl paraben in 1% Na_2CO_3 solution of 0.005 Gm./L. Sodium carbonate solution was used to convert the butyl paraben to the sodium salt of its enolic form and thus shift its absorption curve to the right of the caffeine absorption curve. The peak absorption for butyl paraben in sodium carbonate solution was at 296 mμ, yielding a specific extinction coefficient of 117.6 which was used for subsequent determinations of the ester in the presence of caffeine.

The experimental procedure was the same as that for aspirin except that 0.20 Gm. of butyl paraben at 15° and 0.30 Gm. at 30° was used as excess. The bottles were shaken for fifteen hours and the solutions were then analyzed spectrophotometrically for the ester. The final dilution prior to analysis was made with a 1% Na_2CO_3 solution.

Methods for Correction of Caffeine Absorption.—Two methods were employed with good results.

The optical density of caffeine, 0.060 Gm./L., was determined at 296 mμ. The specific extinction coefficient value of 2.516, obtained at this wave length and caffeine concentration, was used to calculate the optical density for caffeine. This value was subtracted from the optical density obtained for the butyl paraben-caffeine solution.

Alternately, a caffeine blank, diluted like the butyl paraben-caffeine mixture, was used for each determination.

Results.—The saturated concentrations of butyl paraben found in the presence of caffeine are tabulated in Tables X and XI. The equilibrium constants for this reaction were determined by assuming the interaction to be of the following form:

$$\text{Caffeine} + \text{Butyl Paraben} \rightleftarrows [\text{Caffeine-Butyl Paraben}] \quad (6)$$

TABLE X.—BUTYL PARABEN AND CAFFEINE IN AQUEOUS SOLUTION AT 15°

Orig. Caffeine Concn., × 10^2 M	Paraben Concn., × 10^2 M
0.000	0.644
0.249	0.736
0.520	0.858
0.763	0.950
1.021	1.081
1.299	1.222
1.659	1.419
2.026	1.602
2.347	1.769
1.030	1.051
2.060	1.567
3.090	2.119
4.120	2.601
5.150	3.183
5.152	3.126
6.180	3.695
7.215	4.422[a]
8.239	5.053[a]
9.271	5.122[a]

[a] Blank was saturated with respect to caffeine.

TABLE XI.—BUTYL PARABEN AND CAFFEINE IN AQUEOUS SOLUTION AT 30°

Orig. Caffeine Concn., × 10^2 M	Paraben Concn., × 10^2 M
0.000	1.305
1.030	1.913
2.060	2.474
3.090	2.986
4.120	3.669
5.150	4.168
6.180	4.816
7.214	5.306
8.239	6.065
9.269	6.653
10.322	7.399
11.334	7.837
12.870	8.844
13.906	9.588
14.973	11.031[a]
16.543	12.784[a]

[a] Blank saturated with respect to caffeine.

The equilibrium constant for this reaction can be written as:

$$K_e = \frac{[\text{Caffeine-Butyl Paraben}]}{[\text{Caffeine}]\,[\text{Butyl Paraben}]} \quad (7)$$

Since a straight line relationship was obtained for the solubility of butyl paraben in the presence of caffeine (Fig. 7), the equilibrium constants were readily determined at the two temperatures on the basis of Eq. 7, as follows:

Solubility of butyl paraben.....	$0.58 \times 10^{-3} M$
Original caffeine concn.....	$6.25 \times 10^{-3} M$
Butyl paraben concn.....	$3.72 \times 10^{-3} M$
Complex concn., $3.72 \times 10^{-3} - 0.58 \times 10^{-3}$	$= 3.14 \times 10^{-3} M$
Free caffeine remaining, $6.25 \times 10^{-2} - 3.14 \times 10^{-3}$.....	$= 5.94 \times 10^{-2} M$

Therefore according to Eq. 7, the equilibrium constant at 15° is:

$$K_e = \frac{3.14 \times 10^{-3}}{5.94 \times 10^{-2} \times 0.58 \times 10^{-3}} = 91$$

Similarly, the K_e value at 30° was found to be 50.

Thermodynamic Calculations.—Thermodynamic values, determined in the same manner as for benzoic acid, are tabulated in Table XII.

Discussion.—Results indicate that caffeine and butyl paraben interact to form at least one type of complex. This conclusion is supported mainly by the fact that there is a straight line relationship for the solubility of the butyl paraben and the caffeine added, up to the point of saturation with respect to caffeine. The high degree of interaction, evidenced by the high entropy change and heat of reaction, indicates that the ester group takes an important part in the complex formation.

GENERAL DISCUSSION

The thermodynamic constants for all of the compounds studied are compared in Table XIII. These data show that caffeine forms rather stable complexes with all the compounds investigated.

In all cases the predominant complex contains one molecule of caffeine and one of acid. In addition there is strong evidence for the formation of complexes containing two molecules of acid to one of caffeine, e.g., in the case of benzoic acid, and also for complexes formed from two molecules of caffeine to one of acid, e.g., in the case of salicylic acid and salicylate ion. At present conclusive evidence for formation of complexes containing more than three molecular units per complex is lacking.

The position of functional groupings seems to influence the stability of the complex. For example, the *para* form of hydroxybenzoic acid has much

TABLE XII.—SUMMARY OF RESULTS FOR BUTYL PARABEN-CAFFEINE INTERACTION

Temp., °C.	K_c	Slope of Line Log K_c vs. $1/T$	ΔH, Cal./Mole	ΔF, Cal./Mole	ΔS, Cal./Deg./Mole
15	91	1530	−7000	−2600	−15
30	50	1530	−7000	−2370	−15

TABLE XIII.—SUMMARY OF RESULTS

Complex	Temp., °C.	Equil. Constant	$\Delta F°$, Cal./Mole	ΔH, Cal./Mole	ΔS, Cal./Deg./Mole
Caffeine-benzoic acid	0	29	−1900	−3000	−4
	15	23	−1800	−3000	−4
	30	18	−1800	−3000	−4
Caffeine-(benzoic acid)$_2$	0	12	−1350	−4000	−11
	15	7.5	−1150	−4000	−11
	30	5.5	−1050	−4000	−11
Caffeine-benzoate	0	23.1	−1700	−3300	−6
	15	16.4	−1600	−3300	−6
	30	12.8	−1540	−3300	−6
Caffeine-aspirin	15	19.8	−1720	−1500	0.6
	30	17.4	−1730	−1500	0.6
Caffeine-salicylic acid	15	...[a]	...	...	...
	30	44	...	...	...
Caffeine-salicylate ion	15	(?)[b]	...	...	...
	30	(?)[b]	...	...	...
Caffeine—*p*-hydroxy-benzoic acid	15	100	...	...	...
	30	100	...	...	...
Caffeine-*m*-hydroxy-benzoic acid	15	....[a]	...	...	...
Caffeine-butyl paraben	15	91	−2600	−7000	−15
	30	50	−2370	−7000	−15

[a] Indeterminate.
[b] Determination doubtful because of formation of higher complexes.

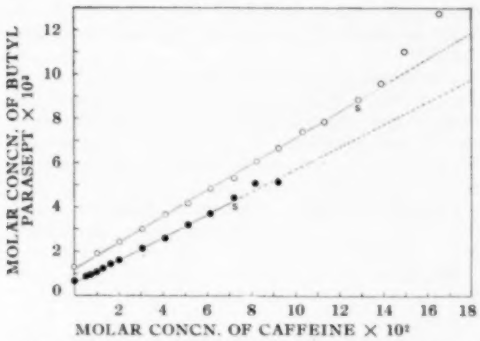

Fig. 7.—Solubilizing influence of caffeine on butyl parasept. ●——● at 15°; o——o at 30°. *S* indicates saturation with respect to caffeine. The straight line functions relate the solubility of the paraben to caffeine concentration.

greater complexing ability than the *ortho*.. This is not surprising since the *ortho* structure permits greater self-satisfaction of the complexing tendencies of the hydroxyl and the carboxyl groups than does the *para* form. This difference is also probably responsible for the higher melting point, greater water solubility, and lower vapor pressure of the *para* acid as compared to the *ortho*. The *meta* isomer partakes apparently more of the characteristics of the *para* than of the *ortho*.

In general, the loss of a hydroxyl hydrogen by esterification seems to result in a lowered complexing tendency, shown by the lower stability of the complex formed from acetyl salicylic acid as compared to salicylic acid and also in the case of butyl paraben as compared to *p*-hydroxybenzoic acid.

The magnitude and the sign of the thermodynamic constants for the complexing reaction are in agreement with what might be expected. The heats of reaction range from 1.5 to 7 kg.-cal., averaging little less than 4. These energies probably represent the net changes accompanied by formation and disruption of several hydrogen bonds and are comparable in magnitude with those involved in enzymatic reactions. The entropy changes show wide variations but correlate well with the corresponding heats of reaction. In general, higher heats of reaction are accompanied by greater loss in entropy or, equivalently, by a greater degree of ordering in the final complex.

It is apparent from the preceding discussion that there is still much to be learned concerning the nature of the complexes formed by caffeine and other xanthines. Many more compounds must be studied and stability constants determined before such questions as the points of attachment, the nature of the chemical bond, and the stereochemistry of the complexes can be answered. The complexity of the problem is indicated by the fact that Debye and London forces seem to take prominent part in the over-all interaction. The evidence for the dipole-induced dipole reaction lies in the ready solubility of caffeine in benzene and other aromatic hydrocarbons as compared to diethyl ether.

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Determination of Morphine in Opium with the Aid of an Adsorption Column*,†

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An assay of morphine in opium is proposed which utilizes both adsorption and colorimetric procedures.

SINCE the discovery of morphine in 1803 by Sertürner (9), a great deal of literature on the analysis of opium has appeared. The assay of opium given in the majority of pharmacopeias is by either the Helfenberger or the lime method (12), and most of the literature deals with modifications of these methods. Methods utilizing polarography (11), colorimetry (1, 4, 5, 13, 14, 16, 17), and ion-exchange (10) have also been reported. Adsorption procedures on opium (3, 6) have been carried out, using alumina as the adsorbent.

The official opium assay, which is extremely long and tiring, is believed to give low results, which Rosin and Williams (12) found to be due to various factors such as the loss of the alkaloid in the solvent and incomplete liberation from lime. Ammonium chloride, used to precipitate the morphine in the last step of the U. S. P. method, will also dissolve some of the alkaloid. They also noticed that results differed when there was a variation in the temperature at which the formation of morphine crystals takes place. Another disadvantage of the official assay, observed in this laboratory, is the adsorption of interfering colored extractive material on the precipitated morphine crystals, which makes the end point in titration rather difficult to see.

In this investigation, a short, simple, and direct one-solvent extraction (using a minimum amount of solvent), an adsorption column to remove interfering material, and a final colorimetric determination of morphine were employed.

EXPERIMENTAL

The elution apparatus of Stolman and Stewart (15), described in their work with morphine, codeine, and heroin in viscera and body fluids by adsorption, was employed. It was found that extraction and elution could be accomplished at one time by lengthening this apparatus and placing a glass thimble above the adsorption column (Fig. 1).

A number of reagents were tested for the colorimetric morphine determination. The Folin-Ciocalteu (7) reagent, previously used by Stolman and Stewart, was found to give the best results.

Preparation of Reagents

The Folin-Ciocalteu Reagent.—One hundred grams of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, and 25 Gm. of sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, are dissolved in 700 ml. of water in a 1,500-ml. Florence flask. Fifty milliliters of 85% phosphoric acid and 100 ml. of concentrated hydrochloric acid are added, the flask is connected to a reflux condenser, and the mixture is boiled gently for ten hours. It is imperative to use glass-stoppered equipment in order to prevent decomposition of the reagent. After the heating period, 150 Gm. of lithium sulfate, $Li_2SO_4 \cdot H_2O$, is added, followed by 50 ml. of distilled water and a few drops of liquid bromine. The mixture is boiled without the condenser for fifteen minutes to remove the excess bromine. After cooling, the reagent is diluted to 1 L. and, if necessary, is filtered rapidly.

A greenish tint in the reagent indicates the presence of blue reduction products which lessen the range of true proportionality between different small amounts of morphine. If the reagent is exposed too often to the air, any organic material present gradually produces slight reduction.

Saturated Sodium Carbonate Solution.—$Na_2CO_3 \cdot H_2O$, C. P., was added in excess to distilled water, allowed to stand overnight, and filtered.

Morphine Stock Solution.—Morphine was purified by recrystallization from absolute methanol.

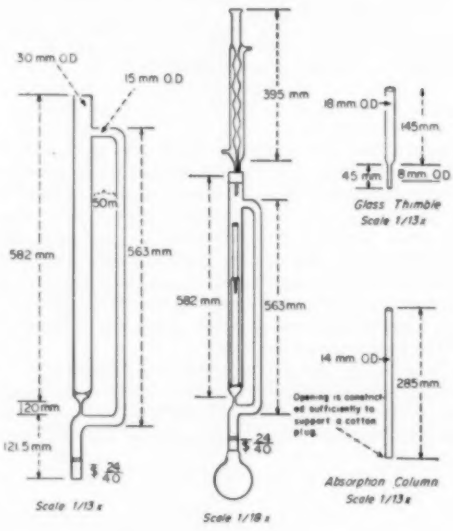

Fig. 1.—Slightly modified Stolman-Stewart apparatus.

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In order to compensate for any remote possibility of a change due to the treatment of morphine as applied to the final assay of opium, the morphine used in the standard solutions is prepared as follows:

A portion of 100 mg. of pure morphine is transferred to a 125-ml. Erlenmeyer flask. After the addition of 50 ml. of absolute methanol and a few glass beads, the solution is refluxed for three hours. The morphine solution (and glass beads) is transferred quantitatively with absolute methanol to an evaporating dish, and is evaporated to dryness. While the evaporating dish is still fairly warm, about 5–10 ml. of dilute hydrochloric acid is added and the morphine is dissolved by stirring. The solution is filtered into a 100-ml. volumetric flask and is brought to volume with distilled water. This stock solution contains 1 mg. of morphine per ml. of solution.

A standard morphine solution with a concentration of 0.1 mg. (100 μg.) of morphine per ml. of solution, is prepared by diluting 10 ml. of the stock solution to 100 ml.

Procedure for the Colorimetric Test.—To obtain a standard curve, aliquots of a morphine solution representing 100–400 μg. of the alkaloid are transferred to 100-ml. volumetric flasks. Two milliliters of the Folin-Ciocalteu (7) reagent is added, followed by 3 ml. of the saturated sodium carbonate solution and enough distilled water to bring the volume to 100 ml.

After five hours, the maximum blue color develops and is stable for at least eighteen hours. The color readings, which follow the Beer-Lambert law, can be made in any suitable instrument at 765 mμ. In this study, the instrument used was a Coleman Model 11 spectrophotometer. For the blank the morphine solution is omitted. Figure 2 shows a typical absorption curve.

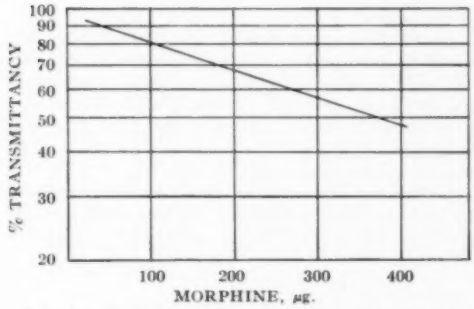

Fig. 2.—A typical absorption curve of the blue-colored solutions obtained in the colorimetric test for morphine. Transmittance readings of the solutions are plotted against their concentrations. The wave length used was 765 mμ.

Specificity of the Reagent.—Some codeine and narcotine solutions gave a positive test with the Folin-Ciocalteu reagent, while other samples did not. Stolman and Stewart have reported that the test was not positive with pure codeine solutions (15). The positive test was probably due to contamination by morphine. Synthetic papaverine did not give the test. The fact that narcotine, codeine, and papaverine contain no phenolic group explains the lack of a positive test with the reagent.

Meconic acid, an organic acid found in opium, gave a blue color with the reagent, but when it was treated by the suggested opium assay, it was adsorbed on the Florisil column and did not interfere with the colorimetric determination (Tables II and IV).

Selection of Adsorbent.—Of the various columns of lime, magnesium oxide, Amberlite IR-4B, alumina and Florisil,[1] the latter was found to be the most efficient adsorbent and the most convenient to use: (*a*) it does not require admixture with a filter aid; (*b*) a preliminary preparation by chemical treatment is not needed prior to use; (*c*) it is easily and evenly packed in a column.

Assay of Opium

Reagents.—Florisil, 60–100 mesh; absolute methanol, C. P.; Folin-Ciocalteu reagent; saturated sodium carbonate solution.

Adsorption Column.—A small wad of cotton is fitted into the bottom of a glass tube 285 mm. in height and 12 mm. in inner diameter. Florisil is packed to a height of 200 mm. into this glass tube, and another piece of cotton is placed on top of the Florisil. About 40 ml. of absolute methanol is passed through the column, and at the same time a glass rod with a diameter slightly less than that of the glass tube is used to press out any air pockets that might have formed between the cotton and the Florisil.

Extraction, Elution, and Determination of Morphine.—An opium sample (0.5 Gm.), previously dried to constant weight at a temperature not exceeding 70°, is weighed into a folded, cone-shaped, 9-cm. filter paper. The opium is covered with cotton and the sample is carefully placed in the base of a glass thimble 145 mm. in length and 18 mm. in outer diameter. The stem of the thimble, about 45 mm. in length and 8 mm. in outer diameter, extends into the adsorption column when the glass thimble is in place over the column. A 500-ml. flask contains 100 ml. of absolute methanol (and a few glass beads) for the extraction and elution of morphine. The entire apparatus is assembled as shown in Fig. 1.

The solvent is boiled at a rate adjusted to ensure a constant small layer of methanol above the Florisil. The rate of percolation through the column at the start is very slow, but it increases within a short time. The methanol layer above the Florisil is regulated so that it does not overflow into the 500-ml. flask.

After three hours of refluxing (Table I), the opium extract is transferred quantitatively (using absolute methanol) to an evaporating dish and evaporated to dryness on a steam bath; the residue is allowed to cool. About 30 ml. of distilled water is added to the residue and the evaporating dish is swirled to loosen the residue. The swirling is necessary in order to prevent the formation of a colloidal suspension of the resinous material which makes filtration difficult. The solution is quantitatively transferred and filtered into a 200-ml. volumetric flask; distilled water is used to rinse the evaporating dish and to adjust the final volume of the solution.

One milliliter of this opium extract, representing approximately 250 μg. of morphine, is transferred to

[1] Floridin Co., Warren, Pa.

TABLE I.—DETERMINATION OF EXTRACTION TIME FOR THE COMPLETE RECOVERY OF MORPHINE

Time, Hr.	Wt. of Morphine Sample, Mg.	Morphine Found, Mg.	Recovery, %
2	51.0	50.0	98.04
2	50.5	49.8	98.62
3	50.8	51.0	100.4
3	49.9	49.8	99.80
3	50.3	50.6	100.6
4	41.9	41.8	99.76

TABLE II.—MECONIC ACID ADDED[a]

Meconic Acid, Mg.	Wt. of Morphine Sample, Mg.	Morphine Found, Mg.	Recovery, %
50.0	48.5	48.2	99.38
50.0	49.2	49.0	99.59

[a] Meconic acid with morphine treated according to the proposed assay procedure (three-hour heating period).

a 100-ml. volumetric flask and treated in the same manner as the pure morphine solutions.

The samples of opium cannot be larger than 0.5 Gm. when the size of column specified and the three-hour extraction period are used. The final volume of opium extracts, containing a high percentage of morphine, is adjusted so that 250 μg. of morphine will be contained in an aliquot of not less than 1 ml. Tables IV, V, and VI shows the results on three different lots of opium, using the proposed assay.

Interferences.—The entire procedure was carried out without an opium sample, and the results show that neither the adsorbent nor the solvent produces any interference.

It was observed further that the adsorption column removes some interfering colored substances, because without the use of the column, the percentage of morphine found is apparently doubled (Tables III and IV).

TABLE III.—THREE HOURS OF REFLUXING WITHOUT COLUMN

Wt. of Opium Sample, Gm.	Morphine Found, Mg.	Recovery, %[a]
0.4896	114.5	23.39
0.4870	112.5	23.10

[a] The percentage recovery actually includes interferences.

Opium extracts obtained in the proposed assay are amber colored. Ether was used to remove the color from these aqueous extracts, and the morphine was again determined. No differences in the results were observed, indicating the absence of any color interference.

Due to the fact that most pharmaceutical companies use lactose as a diluent for opium, this compound was tested with the Folin-Ciocalteu reagent. Lactose in the amount of 25–35% of the weight of an opium sample was dissolved in water and made up to 200 ml. An aliquot of 1 ml. was tested colorimetrically in the same manner as morphine. No interference was noted.

When a sample of lactose was treated in the same manner as an opium sample, the results were the same as those obtained with unheated lactose. If the heating period is increased to five hours, more lactose is dissolved and the color of the solution darkens and interferes with the colorimetric determination. Limiting the extraction period of the opium assay to three hours eliminates this interference and insures complete extraction of the morphine.

DISCUSSION

An assay of morphine is proposed which is based on the fact that morphine can be extracted from opium with boiling methanol and then eluted from a Florisil column with the same solvent, while interfering materials such as meconic acid and colored substances are adsorbed.

The colorimetric test used in this work has been shown to be fairly specific, because with the exception of morphine, pure solutions of the more abundant alkaloids of opium do not give a test with the Folin-Ciocalteu reagent. Thus these alkaloids will not interfere if they are eluted with morphine. Furthermore, the color reagent will not give a test with meconic acid or lactose under the conditions of the assay.

Borke (2), using surface chromatography, has found that morphine and papaverine were the only alkaloids present in extracts prepared according to the proposed method. Since it was shown that papaverine does not develop a color with the Folin-Ciocalteu reagent, it is fairly certain that only morphine was determined.

Studies by Folin and Denis (8) on their reagent, which is basically the Folin-Ciocalteu reagent, show that the active compound in the reagent is probably

TABLE IV.—RESULTS OF LOT A-1 OPIUM (PROPOSED ASSAY)

Meconic Acid, Mg.	Morphine Added, Mg.	Wt. of Opium, Gm.	Total Morphine Found, Mg.	Morphine in Sample, Mg.	Morphine in Sample, %
0	0	0.4682	54.80	54.80	11.70
0	0	0.4841	56.80	56.80	11.73
0	0	0.4820	56.20	56.20	11.66
0	0	0.4730	55.40	55.40	11.71
0	0	0.4899	57.60	57.60	11.76
0	12.1	0.4916	68.13	56.03	11.40
0	19.3	0.4888	77.33	58.03	11.87
0	18.2	0.4901	74.50	56.30	11.49
0	19.1	0.4860	75.58	56.48	11.62
20.0	0	0.4721	57.00	57.00	12.07
10.0	10.1	0.4776	65.00	54.90	11.50
10.0	16.5	0.4684	72.33	55.83	11.92

reduced by phenols in acid solution and that the reduced compound forms blue salts on the addition of the alkali. The blue color is not very stable in an excess of alkali because it gradually fades and the more concentrated the alkali, the more rapidly it fades. It was observed during the present investigation that 2 ml. of the Folin-Ciocalteu reagent, followed by 3 ml. of the alkali (saturated sodium carbonate solution), gives the best reproducible results.

Several milligrams of meconic acid, whether tested before or after refluxing, without the Florisil column, give a strong positive test with the Folin-Ciocalteu reagent. However, when larger portions of meconic acid are extracted according to the proposed method, a positive test is no longer obtained. Tables II and IV show that meconic acid is adsorbed on the Florisil.

A three-hour refluxing period is used in the proposed assay, because the experimental evidence, shown in Table I, indicates that this period is necessary for complete recovery of a 50-mg. sample of morphine. A longer period of refluxing is not advocated because lactose, the diluent in opium, undergoes a browning reaction and produces an interfering color (Table VII).

The proposed assay (Tables IV, V, and VI) gives higher results than the official method (Table VIII). This may be accounted for in one or more of the following ways: (*a*) less chance for loss of morphine since there are fewer manipulations; (*b*) the absence of lime and ammonium chloride; (*c*) complete precipitation of morphine; and (*d*) a colorimetric determination rather than a titration method.

When weighed amounts of pure morphine are added to opium samples prior to extraction, recoveries are excellent (Tables IV, V, and VI).

TABLE V.—RESULTS OF LOT A-2 OPIUM (PROPOSED ASSAY)

Morphine Added, Mg.	Wt. of Opium, Gm.	Total Morphine Found, Mg.	Morphine in Sample, Mg.	Morphine in Sample, %
0	0.4861	66.40	66.40	13.66
0	0.4916	69.20	69.20	14.08
0	0.4890	67.00	67.00	13.70
0	0.4909	68.60	68.60	13.97
0	0.4885	68.40	68.40	14.00
18.6	0.4937	87.66	69.06	13.99
18.7	0.4880	86.00	67.30	13.79
18.1	0.4820	84.67	66.57	13.81
17.0	0.4940	86.33	69.33	14.03

TABLE VI.—RESULTS OF LOT A-3 OPIUM (PROPOSED ASSAY)

Morphine Added, Mg.	Wt. of Opium, Gm.	Total Morphine Found, Mg.	Morphine in Sample, Mg.	Morphine in Sample, %
0	0.4903	65.40	65.40	13.34
0	0.4962	66.00	66.00	13.30
0	0.4888	67.00	67.00	13.71
0	0.4573	62.40	62.40	13.65
0	0.4858	64.80	64.80	13.34
18.1	0.4933	85.09	66.90	13.56
19.3	0.4908	84.33	65.03	13.25
16.1	0.4942	82.25	66.15	13.39

TABLE VII.—VARYING HEATING PERIOD (PROPOSED ASSAY)[a]

Time, Hr.	Wt. of Opium, Gm.	Morphine Found, Mg.	Morphine in Sample, %
3	0.4682	54.80	11.70
3	0.4841	56.80	11.73
3	0.4820	56.20	11.66
3	0.4730	55.40	11.71
3	0.4899	57.60	11.76
4	0.4902	60.60	12.36
4	0.4932	63.00	12.77
4	0.4904	64.60	13.17
4	0.4889	64.00	13.09
4	0.4940	68.00	13.77
5	0.4683	62.25	13.29
5	0.4895	68.60	14.01
5	0.4771	66.80	14.00
5	0.4674	68.75	14.71

[a] Lactose interference causes a noted increase in the results when the heating period is longer than three hours.

It is important to use samples of opium no larger than 0.5 Gm., otherwise a longer refluxing period is necessary for complete morphine recovery. A longer heating period must be avoided, because lactose might interfere with the determination.

TABLE VIII.—RESULTS OF THREE LOTS OF OPIUM (U. S. P. ASSAY)

Lot Number	Wt. of Opium, Gm.	Opium in Aliquot, Gm.	Morphine Found, Gm.	Morphine, %
A-1	6.00	3.965	0.3863	9.74
A-1	6.00	3.960	0.3837	9.69
A-1	6.00	3.959	0.3832	9.68
A-1	6.00	3.963	0.3832	9.67
A-2	6.00	3.712	0.3849	10.37
A-2	6.00	3.777	0.3891	10.30
A-3	6.00	3.321	0.3298	9.93
A-3	6.00	3.500	0.3506	10.02

Any desirable number of extractions can be run in one day with a sufficient number of extractors. The color can be developed as soon as the extraction is complete, and the blue color, stable for eighteen hours, can be read in a spectrophotometer on the following morning.

SUMMARY

An assay of morphine in opium is proposed which utilizes both adsorption and colorimetric procedures. Compared to the present official method, this assay is less time-consuming without loss in accuracy and reproducibility.

Under the conditions of the assay, meconic acid, lactose, the adsorbent, the solvent, and the color of the extract do not interfere with the colorimetric test. Pure solutions of narcotine, codeine, and papaverine do not give a positive test with the Folin-Ciocalteu reagent.

The proposed method gives higher results than the official method; reasons for this are presented.

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The Separation of Morphine from Codeine by Means of Ion Exchange*

By ERNEST W. GRANT and WAYNE W. HILTY

Morphine can be separated from codeine by a strongly basic, quaternary amine-type anion exchanger. The morphine base, due to its phenolic structure, exchanges with the resin while the codeine base does not. Codeine is washed through the column with the solvent and is determined by titration. Morphine is then qualitatively removed from the column with dilute acid and determined spectrophotometrically.

THE increasing value of ion exchange techniques in analytical chemistry has led many workers to study these methods in an effort to develop assay procedures for complex pharmaceuticals and natural products. Several ion exchange methods (1–3) have been described for vitamin preparations and other pharmaceutical applications.

Jindra (4) has developed a rapid, simple method for the determination of alkaloidal salts, using a column of Amberlite IR-4B. The method was reported to be applicable to strychnine nitrate, atropine sulfate, morphine hydrochloride, brucine hydrochloride, ephedrine sulfate, quinine, and cinchonine. In a subsequent report, Jindra and Pohorsky (5) adapted the method to the determination of the total alkaloidal content of cinchona, ipecac, nux vomica, belladonna, and hyoscyamus; no attempt was made to separate or isolate the individual alkaloids.

It was the purpose of this study to determine if quantitative alkaloidal separation could be accomplished by means of ion exchange procedures with the possibility of adapting such a method to galenicals. Morphine and codeine were the alkaloids selected for this study, since present methods for their determination are time-consuming and tedious, and would benefit from any simplification that ion exchange techniques might offer. In this preliminary report, a method for the separation and determination of these two alkaloids is presented.

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EXPERIMENTAL

Principles of the Method.—Morphine, because of its phenolic structure, forms soluble compounds with fixed alkalies while nonphenolic codeine does not. This difference, utilized in the regular chemical separations of these two alkaloids, is the basis of the ion exchange separation. A strongly basic, quaternary amine-type anion exchanger, whose action in the separation is analogous to that of a fixed alkali, was used. The phenolic morphine combines or exchanges with the resin while codeine does not. The codeine base is quantitatively removed from the column by elution with methanol and is then determined by titration. The morphine is subsequently removed by elution with dilute acid and determined spectrophotometrically. The reactions involved may be shown by the following equations:

Codeine: Res. OH + Alk. H_3PO_4 → Res. PO_4 + H_2O + Alk. Base

Morphine: Res. OH + Alk. H_2SO_4 → Res. SO_4 + Res. Alk. + H_2O

Res. Alk. + H_3PO_4 → Res. PO_4 + Alk. H_2PO_4

Reagents and Apparatus.—Of the several anion exchange resins investigated, the most consistent recoveries were obtained with Amberlite XE 75. The resin was converted to the hydroxyl form by allowing it to stand overnight in a 10% aqueous solution of sodium hydroxide, and then decanting the supernatant alkali. The resin was washed with successive portions of distilled water until the rinsings were neutral to phenolphthalein, after which it was placed in a tightly packed column approximately 25 cm. in length.

The morphine sulfate and codeine phosphate used in this study were commercial crystalline samples of U. S. P. quality. Mayer's reagent and Wagner's reagent were used in the general qualitative tests for alkaloids. The potassium ferricyanide-ferric chloride reagent was employed as a specific test for morphine.

Determination of Morphine.—In the method described by Jindra (4), the concentration of morphine in the eluate was determined by titration of the free base. Titration was not possible in the present procedure because the morphine was washed from the column with an excess of acid and was present in the eluate as a salt. The methods described by Adamson (6) and Oram (7) were investigated in an attempt to find a suitable colorimetric method. However, none of these procedures gave satisfactory results in the concentration range desired.

Further investigation led to the development of a convenient and rapid ultraviolet absorption method for the determination of morphine based on the fact that acidified aqueous solutions of morphine sulfate have an absorption maximum at 285 mμ. Standard aqueous solutions of crystalline morphine sulfate were prepared in the range of 1–5 mg. per 25 cc. of solution containing 0.2 cc. of sulfuric acid. The absorbancy was measured at 285 mμ with a Beckman Model DU spectrophotometer, using matched 1-cm. silica cells. The calibration curve obtained under these conditions rigidly followed Beer's law, and a $E^{1\%}_{1cm.}$ value of 39.8 was obtained.

In order to determine the recovery of alkaloid equivalent to morphine sulfate in the eluates, a 10-cc. aliquot was transferred to a 25-cc. volumetric flask, 0.2 cc. of sulfuric acid was added, and the solution was made to the mark with distilled water. The absorbancy of this dilution was read at 285 mμ, and the morphine sulfate concentration of the eluate was determined from a prepared calibration curve (Fig. 1).

Determination of Codeine.—Inasmuch as codeine was present in the eluates as the free base, it was determined by titration. Fifty cubic centimeters of distilled water was added to a 20-cc. aliquot of the eluate and the solution was titrated with 0.01 *N* sulfuric acid, using methylene blue-methyl red mixed indicator. An aliquot of 20 cc. of the methanol used in washing the column was titrated in the same manner for a reagent blank.

Exchange of Morphine Sulfate with XE 75.—The column prepared as described was washed with 100 cc. of distilled water before use. An additional 50 cc. of distilled water was passed through the column and the eluate was retained for a reagent blank in the morphine determination. A sample of 0.2110 Gm. of morphine sulfate was dissolved in 15 cc. of distilled water and the solution was passed through the column. The column was then washed with 300 cc. of distilled water; qualitative tests indicated that there was no morphine or sulfate present in the eluate. The column was then washed successively with 200 cc. of methanol, 200 cc. of ethanol, and 200 cc. of acetone. Tests for morphine and sulfate were negative in all the above eluates. The flow rate in the above washings and in subsequent determinations was 0.12 cc./cc. of resin/min.

The column was then washed with 125 cc. of 2% phosphoric acid, followed by distilled water until the combined eluates measured 250 cc. An aliquot of 10 cc. was taken for spectrophotometric analysis, and a 10-cc. aliquot of the aqueous eluate described served as a reagent blank. A recovery equivalent to 0.2105 Gm. of morphine sulfate was obtained. The column of resin was washed with an additional 50 cc. of 2% phosphoric acid but no morphine was present in the eluate. The experiment was then repeated. Results, tabulated in Table I, indicate that morphine sulfate quantitatively exchanged with the resin, was not removed by aqueous or alco-

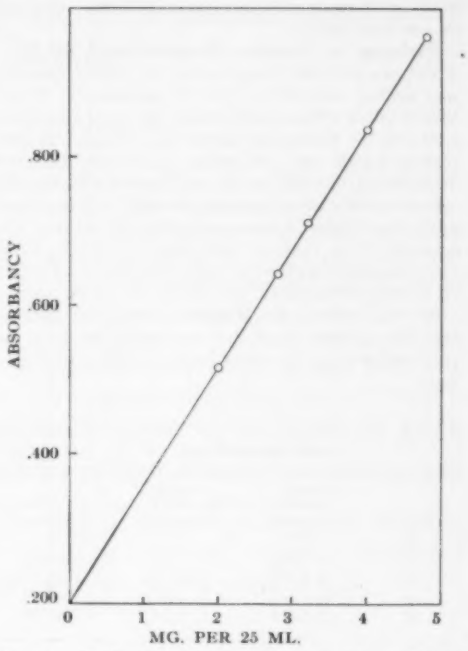

Fig. 1.—Calibration curve of morphine sulfate U. S. P. at 285 mμ.

TABLE I.—EXCHANGE OF MORPHINE SULFATE WITH AMBERLITE XE 75

Sample No.	Weight of Morphine Sulfate, Gm.	Morphine Recovery as Sulfate, Gm.	Recovery, %
1	0.2110	0.2105	99.80
2	0.1243	0.1243	100.00
3	0.1267	0.1269	100.20
4	0.1194	0.1188	99.55
5	0.1026	0.1012	98.60

holic washings, and was quantitatively removed by the acid wash.

Exchange of Codeine Phosphate and XE 75.—A column of resin, prepared in the usual manner, was washed with 250 cc. of 95% methanol. It was then washed with an additional 50 cc. of the methanol and the eluate was saved for a blank. A solution of 0.1800 Gm. of codeine phosphate dissolved in 15 cc. of distilled water was passed through the column, which was then washed with 95% methanol until the combined eluates measured 100 cc. Aliquots of 20 cc. each of this eluate and of the blank were titrated; a recovery equivalent to 0.1807 Gm. of codeine phosphate was obtained. The experiment was repeated. Results (Table II) indicate that the codeine base did not exchange with the resin and was quantitatively eluted by the methanol wash.

TABLE II.—EXCHANGE OF CODEINE PHOSPHATE WITH AMBERLITE XE 75

Sample No.	Weight of Codeine Phosphate, Gm.	Codeine Recovery as Phosphate, Gm.	Recovery, %
1	0.1800	0.1807	100.31
2	0.1169	0.1178	100.71
3	0.1073	0.1082	100.81
4	0.1318	0.1306	99.10

Separation and Determination of Codeine Phosphate and Morphine Sulfate.—A column of resin was prepared, washed with 250 cc. of 95% methanol, and further washed with an additional 50 cc.; the eluate was saved for a blank in the codeine determination. A mixture of 0.1345 Gm. of morphine sulfate and 0.1635 Gm. of codeine phosphate was dissolved in 20 cc. of distilled water, and the solution was passed through the resin column. The column was washed with 95% methanol until a combined eluate of 100 cc. was obtained. An aliquot of 20 cc. of this eluate and the methanol blank were titrated and a recovery equivalent to 0.1629 Gm. of codeine phosphate was obtained. The column was washed with an additional 50 cc. of solvent but no codeine was found in the eluate.

The column was then washed with 100 cc. of distilled water to displace the methanol, and an additional 50 cc. of aqueous eluate was collected to serve as a blank in the spectrophotometric determinations of morphine. The column was then washed with 125 cc. of 2% phosphoric acid followed by distilled water until the combined eluate measured 250 cc. An aliquot of 10 cc. of this eluate and the aqueous blank were taken for analysis and a recovery equivalent to 0.1358 Gm. of morphine sulfate was obtained. This procedure was repeated. Results (Table III) indicate that codeine does not interfere with the exchange between morphine and Amberlite XE 75. Recoveries of the individual alkaloids are consistent with those which were obtained in the preceding determinations.

SUMMARY

A method for the separation and determination of morphine and codeine has been described. The method is simple, reproducible, and rapid.

TABLE III.—RECOVERY OF MORPHINE SULFATE AND CODEINE PHOSPHATE FROM AMBERLITE XE 75

Sample No.	Weight of Codeine Phosphate, Gm.	Recovery of Codeine as Phosphate, Gm.	Recovery, %	Weight of Morphine as Sulfate, Gm.	Recovery of Morphine as Sulfate, Gm.	Recovery, %
1	0.1635	0.1629	99.61	0.1345	0.1358	100.97
2	0.0775	0.0784	101.20	0.1058	0.1048	99.05
3	0.1103	0.1114	101.04	0.1147	0.1148	100.08
4	0.1095	0.1096	100.04	0.1225	0.1196	97.76
5	0.0976	0.0967	99.10	0.1184	0.1175	99.32

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A Determination of the Physical Characteristics of Coal Tar Samples*,†

By IRAIDA N. DE MARTIN‡ and GILMAN N. CYR§

A study was made of the variation of physical characteristics of a number of coal tars, all of which conformed to U. S. P. specifications. These characteristics reflect variations in the amount of pitch present. Suggestions are presented for revision of the U. S. P. monograph on coal tar.

MANY dermatologists have reported on the usefulness of coal tar but complain that it does not produce consistent clinical results. This difference in activity has been attributed to the type of coal used in the production of the tar, to the method of production, and even to the procedure used in compounding the prescription containing the substance. This latter suggestion, however, has been refuted by Downing, Ohmart, and DiCicco (1). It is now generally accepted that variation in clinical results is due essentially to variability in the constituents of coal tar.

Recently, other products obtained in the destructive distillation of coal tar have become of greater commercial importance; thus this has led tar producers to change their methods of production in order to increase the yield of the particular fraction of interest to them. For this reason, coal tars obtained from different sources are different. These differences are physical as well as chemical in nature. Because coal tar is now considered by producers as a by-product, little attention has been paid by them to the problem of obtaining a coal tar which will adapt itself specifically to medical and pharmaceutical needs.

The monograph for coal tar in the United States Pharmacopeia reflects these inconsistencies in coal tar in that it is so unspecific as to permit any market sample of coal tar to comply with it. Until this monograph is revised to a more specific and detailed one we can expect to meet variations in the clinical effects of coal tar.

In order to provide this more selective monograph it will be necessary to analyze a large number of market samples of coal tar and correlate the clinical effects of these tars with the analytical data. With this information at hand the monograph for coal tar can be constructed in such a manner as to exclude from official recognition any tar which either does or does not possess certain definite physical and chemical characteristics.

The purpose of this paper is to present the results obtained when certain physical tests were applied to samples of coal tar obtained from various producers. In addition to this are also included the results of a few compatibility tests in which these various tars were incorporated with several dermatologic vehicles.

Six samples of coal tar have been used in this investigation. Table I presents the production characteristics of the samples of coal tar investigated.

EXPERIMENTAL

In the selection of the physical tests to be applied to the samples of coal tar, reference was made to A.S.T.M. Standards (2) and to the text on coal tar authored by Abraham (3). Tests were selected on the basis of applicability to, and significance for, the purpose of this study. When necessary, some modifications in the tests were introduced.

U. S. P. Specifications for Coal Tar.—In order to substantiate a statement made here earlier, U. S. P. tests were applied to all coal tar samples used in this study. All experimental samples conformed with the statements made in the monograph.

General Appearance.—All the samples exhibited the shiny black color characteristic of coal tar.

Fluorescence.—Solutions of each of the samples in either chloroform, carbon tetrachloride, carbon disulfide, or benzol showed a green fluorescence under ultraviolet light.

Specific Gravity.—By some authors, the specific gravity is considered the most valuable test of all. It has been shown (4) that the specific gravity of dry tar reflects the percentage of free carbon. Volkmann, Rhodes, and Work (5) indicate that an increase in specific gravity is accompanied by an increase in the amount of suspended phase and in the amount of solvent-insoluble materials in the tar. They show that the suspended phase has a strong effect on the specific gravity of the sample. At the same time they indicate that the surface tension and adhesive properties of the sample can also be related to specific gravity determinations.

Procedure.—The method used is based on that suggested in A.S.T.M. Standards, 1949 (6). The results are given in Table II.

Solubility Tests.—Tar consists of a suspension of fine particles, mostly colloidal in nature (ranging from coarse to ultramicroscopic), in a liquid medium

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TABLE I.—PRODUCTION CHARACTERISTICS OF COAL TAR SAMPLES

Sample No.	Oven Type	Destructive Distillation Temp., ° F.	Source of Coal	% Mixture of Low- and High-Volatile Coal	Temp. of Gas at Tar Collection, ° F.
1[a]	Koppers	2,400	Pa. W. Va.	24 Low 76 High	100
2[a]	Koppers	2,400	Pa. W. Va.	24 Low 76 High	>190
3	Wilputte	1,900	Pa. Ky. W. Va.	14 Low 86 High	90
4	Koppers-Becker Wilputte	1,750	W. Va.	20 Low 80 High	180–190
5	Koppers-Becker	2,400–2,500	Va. W. Va.	16 Low 82 High 2 Coke Dust	400–450 and 100 (pooled)
6	Koppers Koppers-Becker	2,680 (K) 2,630 (K-B)	Pa. W. Va.	35 Low 65 High	85% at 200 10% at 104 5% in elec. precipitators (pooled)

[a] Samples 1 and 2 were from the same producer and differ only at the temperature at which the tar was collected.

TABLE II.—SPECIFIC GRAVITY OF COAL TAR SAMPLES

Sample No.	d_{25}^{25}
1	1.1427
2	1.2092
3	1.1725
4	1.1907
5	1.2146
6	1.1609

TABLE III.—SOLUBILITY OF COAL TAR SAMPLES

Sample No.	Tar Insol. in CS_2, %	Tar Insol. in CCl_4, %	Carbenes, %	"Free Carbon" (Benzol), %
1	3.36	10.76	7.30	6.69
2	7.47	21.78	14.31	12.44
3	6.63	16.41	9.78	10.56
4	11.05	20.57	9.52	14.86
5	10.69	27.27	16.58	16.31
6	8.79	15.21	6.42	11.49

which is the continuous phase consisting of a complex mixture of solid and liquid chemical compounds which form the tar oils (7). This liquid medium exhibits high surface tension. If this surface tension is lowered by the addition of a certain solvent, precipitation is observed. The stability of the tar depends on the surface tension of the whole. A lowering of the surface tension results in the flocculation of a part of the resinous material, which consists of the fine particles in suspension (8).

The solubility of tars in different solvents has been long regarded as an excellent means of detecting their various characteristics. Crude bituminous materials are purchased on the basis of their content of matter insoluble in carbon disulfide (9). Carbon tetrachloride has been suggested as a solvent for differentiating bitumens. Logically these solubility tests were considered to be of value in any standardization or assay of coal tar.

Solubility in Carbon Disulfide and in Carbon Tetrachloride.—The procedure followed is based on that suggested in A.S.T.M. Standards, 1949 (10). Results of this determination are given in Table III.

Percentage of Carbenes.—According to Abraham (11) the term "carbenes" was originally proposed by Richardson. It has been applied to that portion of bituminous substances soluble in carbon disulfide but insoluble in carbon tetrachloride. It has already been pointed out how carbon tetrachloride has been suggested as a solvent for differentiating bitumens. Much has been written on the importance of carbenes in engineering projects. Since they are of possible consideration for pharmaceutico-medical purposes, the calculations were made.

The percentage of carbenes is obtained by taking the difference between the percentages of matter soluble in carbon disulfide and in carbon tetrachloride, respectively. This difference represents the percentage of carbenes. The results of these calculations are shown in Table III.

"Free Carbon" Determination.—There has always been much controversy about the most adequate solvent for use in the "free carbon" determination. Previous reference has been made to the way in which different solvents may act in causing differences in the amount of precipitated material. Duriez (8) and others attribute this phenomenon to differences in the surface tension of the solvents used. Other authorities have attributed these variations to chemical reactions between solvent and tar, as well as to coagulation of the colloidal "solutions" of carbon (12).

The so-called "free carbon" in tars is not elemental carbon itself: it consists essentially of a complex mixture of hydrocarbons of high molecular weight, containing 90.0 to 91.7% carbon, 3.4 to 4.0% hydrogen, 1.0 to 1.2% nitrogen, 2.5 to 3.3% oxygen, and 0.7 to 1.4% sulfur (13).

All methods for free carbon determination are open to the objection that results may vary with the solvent used. The carbon separated is of a different degree of purity in each case. Much opposition has been offered to the methods in use at present.

It is only reasonable to state that the solvent employed in a particular determination should be specified.

It has been stated also that the amount of "free carbon" increases with the time the solvent remains in contact with the tar. The amount of this increase varies with the particular solvent employed. Other factors which influence the amount of "free carbon" are the ratio of the amount of solvent to the amount of tar and the temperature at which the test is conducted.

The free carbon content of tars has been regarded as one of the valuable tests performed on this substance. It is considered a good negative index of tar quality. Much free carbon in tar increases its pitch content. From the pharmaceutical point of view it is also undesirable, as is shown by Ohmart (14) in *Coal Tar Solution, N. F.*, in which he observes the incompatibility caused by precipitation of a "brownish-black granular material and a brown deposit on the inner surfaces of prescription bottles."

The following method, differing only slightly from the one already followed for the solubility determinations, was used.

Gooch crucibles were prepared with a mat approximately 2 mm. in thickness. The pad was washed with water until all small particles of asbestos were removed. The crucibles were dried at 105° ± 5°, placed in a desiccator, and weighed. An amount of material which contained 10.0 Gm. ±0.1 Gm. of the substance was weighed into a 125-cc. Erlenmeyer flask, and 50 cc. of pure benzol was added; the solution was stirred thoroughly and was left in contact with the material for five days. (This maceration was performed with the idea of allowing the increase in precipitated matter to reach a maximum). The solution was then passed through the Gooch crucible without suction until the mat was covered. Then gentle suction was applied to the crucible and the remaining solution added, care being taken that the mat was covered with solution at all times. The flask was rinsed clean with pure benzol and the washings added to the crucible until the descending solvent was colorless. The crucible was dried at 105° ± 5°, cooled in a desiccator, and weighed. The increase in weight is the weight of "free carbon" or the portion insoluble in benzol. The results are presented in Table III.

Viscosity Determination.—Coal tar does not lend itself conveniently to the usual viscosity determinations because of its viscid nature and its opacity, and for other reasons. The variation in viscosity from sample to sample may also be so great as to prohibit the use of one method for all samples of coal tar. For these reasons it was considered advisable in this work to devise a method which would measure as accurately as possible the majority of the coal tar samples available.

The apparatus used consists of a funnel with a short length of rubber tubing attached to the stem. A pinchclamp on the rubber tube serves to start and stop the flow of material. A fixed volume of the material was introduced into the cone of the funnel to fill it to a certain mark. The clamp was released and the time required for 50 cc. of the material to flow out was ascertained by means of a stop watch.

The instrument was standardized with 95% glycerin, U.S.P., sp. gr. 1.249. This was used as a reference material because its viscosity is intermediate among the coal tar samples, and because accurate data on its viscosity are available. The glycerin was submitted to the same tests as the samples, and the results were used to convert the readings of the coal tar samples to poises. The determination was made at 20°. The results of this determination are shown in Table IV.

TABLE IV.—VISCOSITY OF COAL TAR SAMPLES

Sample No.	Viscosity, Poises
1	1.8
2	12,275
3	24.1
4	10.1
5	2,734
6	44.7

Surface Tension Determination.—Because of the importance of surface tension in relation to solubility and other determinations, it was deemed advisable to determine the surface tension of the experimental samples of coal tar. A Cenco-du Nouy Tensiometer was used; the samples were kept at a temperature of 25°. The results are summarized in Table V.

TABLE V.—SURFACE TENSION OF COAL TAR SAMPLES

Sample No.	Surface Tension, Dynes/Cm. at 25°
1	46.18
2	51.25
3	48.09
4	47.56
5	49.78
6	48.71

FORMULATION STUDIES

In a strict sense, this perhaps should not be considered a determination of physical characteristics. However, it is well known that various samples of tar will act differently when incorporated in the commonly used vehicles. In recognition of this, and to show further the difference in coal tars, it was thought advisable to incorporate the coal tar samples in a number of vehicles.

In the selection of vehicles, an attempt was made to include bases representing most of the currently used types. The following bases were used:

A. Washable Ointment Bases
   1. Hydrophilic Ointment, U. S. P. XIV
   2. A vanishing cream[1]

B. Greasy Ointment Bases
   1. Hydrous Wool Fat, U. S. P. XIV
   2. Yellow Ointment, U. S. P. XIV

Each of the samples of coal tar was incorporated in 5% strength in each of the bases, and observations were made. The ointments were compounded with a spatula on a tile.

[1] This base consisted of 15% stearic acid, 1% cetyl alcohol, 0.7% potassium hydroxide U. S. P., 10% glycerin, and water.

Observations on Compatibility of Coal Tar.—Samples No. 2 and 5 produced some ointments in which the tar was coarsely dispersed, showing very fine grains of the tar throughout the ointment. This incompatibility was observed with hydrophilic ointment, the vanishing cream, and to a lesser extent, with yellow ointment. A similar incompatibility was observed between Sample No. 5 and hydrous wool fat.

DISCUSSION

Even though Samples No. 1 and 2 were produced by the same manufacturer, they show a difference in their compounding characteristics. All the production characteristics of these samples are identical except for the condensation temperatures. Sample No. 2 was condensed while the gas was at a temperature of over 190° F., while Sample No. 1 was condensed after the gas had cooled to around 100° F. This, apparently, is responsible for the considerable difference seen in those physical characteristics which measure the amount of "pitch" in a sample. Sample No. 2 shows much higher values for specific gravity, percentage material insoluble in carbon disulfide and in carbon tetrachloride, percentage of carbenes, percentage of free carbon, viscosity, and surface tension. Sample No. 2 did not produce satisfactory ointments in most instances, whereas Sample No. 1 did, which shows definitely that the amount of pitch in coal tar plays an important part in the preparation of satisfactory ointments from coal tar.

Sample No. 5 was also obtained from the gas when the gas was still at a high temperature, and it also shows a very high percentage of pitch. A portion of this tar was condensed when the gas was at a temperature of 400–450° F. The ointments made from this sample were also all unsatisfactory.

It would therefore seem advisable to suggest that the present monograph for U. S. P. coal tar be revised to include a test which would limit the amount of pitch in official coal tar. An examination of the various tables shows that either a determination of carbenes or of viscosity would serve the purpose. In what manner this will affect the clinical effectiveness of coal tar, if at all, however, remains to be determined.

SUMMARY AND CONCLUSIONS

1. It is shown that even though all coal tar samples investigated meet U. S. P. specifications, they do nevertheless exhibit marked differences in certain physical properties. It can be seen that these differences in physical characteristics are related in some manner to the compatibility of the coal tar with some ointment bases.

2. Samples No. 2 and 5 were found to produce inferior ointments when incorporated with Hydrophilic Ointment, U. S. P., Yellow Ointment, U. S. P., or with a vanishing cream base. In addition, Sample No. 5 produced an inferior ointment with Hydrous Wool Fat, U. S. P.

3. Examination of the physical characteristics of Samples No. 2 and 5 reveals that these samples exhibit high specific gravity, low solubility in carbon tetrachloride, a high percentage of carbenes, a very high viscosity, and a slightly elevated surface tension. These measurements reveal that the samples contain a high percentage of pitch. Apparently, this results from condensing the tar when the temperature of the gas is still quite high.

4. It is suggested that the U. S. P. introduce a limit on the amount of pitch present in coal tar, which should greatly improve its compatibility.

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Influence of Electrolytes, *p*H, and Alcohol Concentration on the Solubilities of Acidic Drugs*,†,‡

By TAKERU HIGUCHI, MOHIT GUPTA, and L. W. BUSSE

A study has been made to determine the validities of certain theoretical relationships, based on the law of mass action, which predict the influence of *p*H, electrolyte concentration, and additives such as alcohol on solubilities of weakly acidic pharmaceuticals. Experimental data obtained from sulfathiazole, barbiturates, and terramycin indicate that deviations from the expected relationships are usually small unless complex formation takes place.

IN THE formulation of liquid medicinal preparations, the solubilities of the individual drug constituents are of primary pharmaceutical concern. Any theoretical relationship or concept which can usefully predict equilibrium solubility is of considerable value to those in the formulation field. In the present study, an effort has been made to determine experimentally the validity of certain mathematical equations relating the solubilities of certain acidic drugs to the composition of the solvent.

In the present investigation a study has been made of the influence of apparent *p*H, neutral salt content, and the concentration of ethanol in water-ethanol mixtures on the solubility of sulfathiazole and other weakly acidic, difficultly soluble drugs. The experimental data obtained were used to substantiate the validity of the relationship predicted by the law of mass action.

THEORY

The application of the law of mass action to the prediction of solubility of difficultly soluble, weakly acidic drugs at various *p*H levels has been previously discussed (1, 2). Essentially the relationship can be expressed mathematically as

$$S = S_o + S_o \frac{K_a}{[H^+]} \qquad (1)$$

or

$$\log (S - S_o) = \log S_o - pK_a + pH \qquad (2)$$

This equation is exact, however, only if S and S_o are expressed as activities and if the ionization constant is the thermodynamic value.

The exact relationship can also be written in terms of concentrations by introducing the activity coefficient product.

$$S = S_o + S_o \cdot K_a \frac{fHA}{[H^+]\, fH^+ \cdot fA^-} \qquad (3)$$

In practical work it is extremely inconvenient to make use of activities or activity coefficients. For this reason, an approximate equation in which all of the activity coefficients are taken as one is normally used, i. e., Eq. 2 with concentration values used for S and S_o and the classical pK_a rather than the thermodynamic value. The magnitude of the error introduced by these approximations and the relative reliability of the relationship can be shown only by experimental work. In the present investigation, experimental data have been obtained for this purpose for phenobarbital, barbital, sulfathiazole, and terramycin. For terramycin, the analogous equation applicable to a weakly basic drug (1) was tested.

EXPERIMENTAL

The apparatus shown in Fig. 1 was used for determinations of the solubilities of the drugs studied. It consisted of a small constant temperature bath equipped with twin rotating wheels arranged to hold 24 serum vials of 10-ml. capacity. Each vial

Fig. 1.—Solubility apparatus.

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‡ Abstracted from a thesis submitted by M. Gupta to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

was charged with a definite amount of solvent, together with approximately 10–30% excess of a solid drug; sealed with a multiple dose rubber cap; and allowed to equilibrate for one to eight days. An aliquot of the supernatent solvent was withdrawn with a hypodermic syringe, and the liquid was weighed and analyzed for its drug content. The salt contents of buffered solutions were adjusted by adding potassium chloride to maintain constant ionic strength.

The method of analysis was somewhat different for each drug.

Phenobarbital.—Phenobarbital was determined by microtitration with alcoholic sodium hydroxide, with thymolphthalein as the indicator. It was necessary to extract the drug from the aqueous medium with chloroform after acidifying the solution with 2 drops of concentrated sulfuric acid, because the buffer employed interfered with the assay. The chloroform extract was titrated directly. Preliminary tests on solutions containing known concentrations of the drug gave recoveries showing average absolute deviation of less than 0.4%.

Barbital.—Barbital was determined in the same manner as phenobarbital except that the extraction was carried out with ether rather than chloroform for greater extractive efficiency.

Terramycin.—Terramycin was determined spectrophotometrically on an aliquot of the saturated solution at 268 mμ in 0.1 *M* hydrochloric acid.

Sulfathiazole.—Sulfathiazole was determined directly spectrophotometrically at 283 mμ with 0.1 *M* citrate buffer of *p*H 5 as the solvent.

All drugs except terramycin were recrystallized from water before use.

RESULTS AND DISCUSSION

The influence of *p*H on solubilities of the several drugs studied are shown in Tables I, II, III, and IV and Figs. 2, 3, 4, and 5 where log $(S - S_0)$ has been plotted against *p*H. All of the samples except barbital appear to give strictly linear plots as predicted from Eq. 2. Barbital seems to possess anomalous solubility behavior at higher *p*H values, which may be due to formation of some insoluble complex in this region.

TABLE I.—SOLUBILITY OF PHENOBARBITAL AS A FUNCTION OF pH AT 25°

Final *p*H	Solubility of Phenobarbital, Gm./100 Gm. Satd. Soln.
Unbuffered solution	0.0635
5.89	0.073
6.12	0.075
6.45	0.079
6.64	0.087
6.68	0.088
6.91	0.104
6.99	0.118
7.10	0.129
7.11	0.133
7.15	0.141
7.16	0.160
7.28	0.151
7.30	0.182
7.36	0.196
7.36	0.183

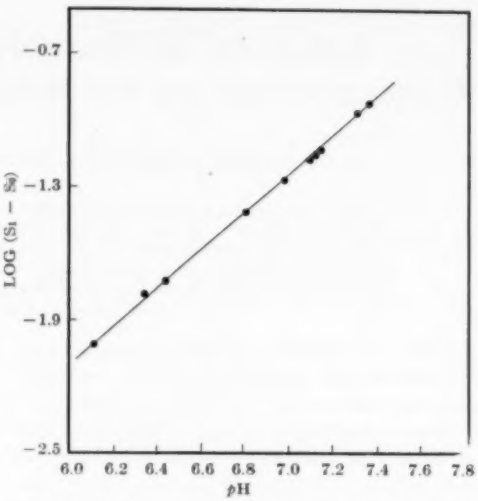

Fig. 2.—Influence of *p*H on the solubility of phenobarbital in water at 25°. Points at *p*H 7.16 and 7.28 were inadvertently omitted from the plot.

TABLE II.—SOLUBILITY OF BARBITAL AS A FUNCTION OF pH AT 30°

Final *p*H	Solubility of Barbital, Gm./100 Cc. Satd. Soln.
Unbuffered solution	0.469
Unbuffered solution	0.506
Unbuffered solution	0.509
Unbuffered solution	0.506
6.04	0.532
6.24	0.572
6.42	0.620
6.66	0.706
6.78	0.784
6.82	0.820
6.91	0.850
7.02	0.915
7.08	0.945

In Table V, the slopes of the straight line plots obtained from the graphs are given. In all cases, the slope values are very close to the unit slope pre-

TABLE III.—SOLUBILITY OF TERRAMYCIN AS A FUNCTION OF pH AT 25°

Final *p*H	Solubility of Terramycin, Gm./100 Cc.
2.12	2.657
2.23	1.911
2.91	0.280
3.48	0.122
3.74	0.074
4.43	0.0325
4.47	0.0360
4.55	0.0361
4.82	0.0314
4.86	0.0486
4.87	0.0259
5.55	0.0276
5.61	0.0300
5.68	0.0297
5.80	0.0313

TABLE IV.—SOLUBILITY OF SULFATHIAZOLE AS A FUNCTION OF pH AT 25°

Final pH	Solubility, Gm./100 Cc. Satd. Soln.	
Dist. Water (I. S. 0.15 *M*)	0.0372	Average—
Dist. Water (I. S. 0.15 *M*)	0.0373	0.0373 Gm./
Dist. Water (I. S. 0.15 *M*)	0.0384	100 cc.
Dist. Water (I. S. 0.15 *M*)	0.0363	Satd. Soln.
6.12	0.0419	
6.17	0.0414	
6.32	0.0443	
6.33	0.0433	
6.48	0.0456	
6.67	0.0500	
6.67	0.0498	
6.85	0.0544	
6.87	0.0540	
7.08	0.0691	
7.08	0.0670	
7.23	0.0756	
7.25	0.0853	
7.28	0.0854	
7.30	0.0858	
7.32	0.0878	
7.38	0.1010	
7.38	0.1010	
7.43	0.1080	
7.43	0.1100	
7.51	0.1210	
7.61	0.1474	
7.51	0.1185	
7.61	0.1450	
7.64	0.1432	
7.64	0.1430	
8.06	0.3220	

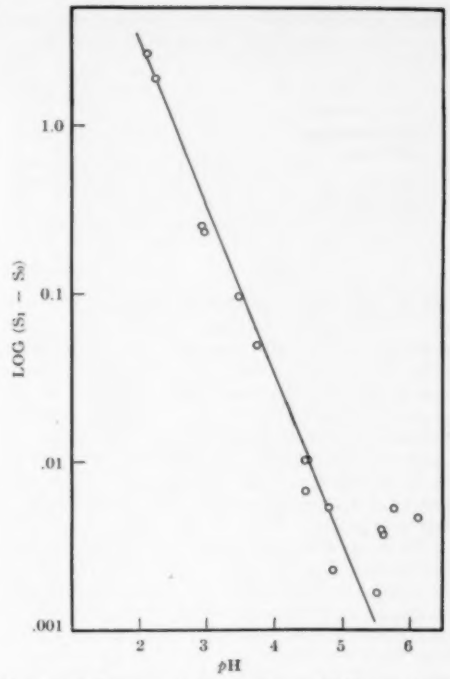

Fig. 4.—Influence of *p*H on the solubility of terramycin at 25° in water.

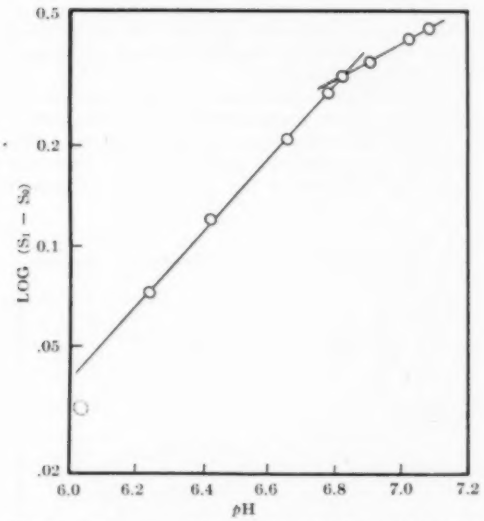

Fig. 3.—Influence of *p*H on the solubility of barbital at 30° in water.

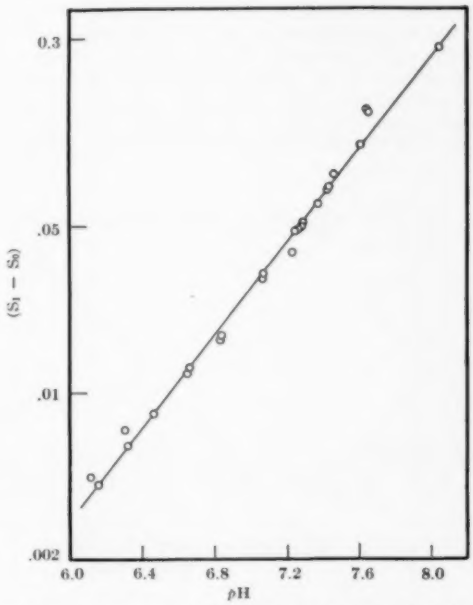

Fig. 5.—Influence of *p*H on the solubility of sulfathiazole at 25° in water.

dicted theoretically. Except for the cationic acid formed from terramycin, the solubility of the acids increased slightly more with *p*H than predicted. For practical purposes, however, it appears that Eq. 2 can be used to predict the solubilities of these and similar drugs at different *p*H values.

In Fig. 6, the influence of alcohol concentrations on the solubility of unionized sulfathiazole in the pres-

TABLE V.—EXPERIMENTALLY DETERMINED SLOPE OF EQUATION 2 FOR SEVERAL COMPOUNDS

Compound	pH Range	Slope, $\frac{\log (S - S_o)}{pH}$
Phenobarbital	6.0–7.4	1.01
Barbital	6.0–6.8	1.05
	6.8–7.1	0.55
Terramycin	2.0–5.0	−0.91
Sulfathiazole	6.0–8.1	1.03

ence of potassium chloride is shown. The near-linear relationship existing between the alcohol concentration and the logarithm of the solubility in the lower alcohol range is somewhat typical of many compounds exhibiting high solubility in alcohol and less in water (3–6). A greater sigmoidal tendency is, however, exhibited in most cases. As a first approximation, a straight line relationship can be expected to exist between the volume fractions of a mixed solvent and the logarithm of its dissolving power. Since the solubility of difficultly soluble drugs is essentially an exponential function of their molecular energies of solution in the absence of a marked entropy effect, the logarithm of solubilities should vary linearly with solvent compositions, if it is assumed that the interaction energies are also linear functions of composition. The latter assumption is valid for nearly ideal solutions. With such nonideal systems as water and alcohol mixtures, however, it is only a crude approximation which leads to some deviation from the linear relationship.

The presence of ionic solute, such as potassium chloride, appears to have little influence on the solubility of sulfathiazole (Fig. 6). With purely nonpolar solute, addition of salts generally results in an increase in the activity coefficient, which tends to lowered solubility. With ionic, zwitterionic, and highly polar substances, on the other hand, an increase in the ionic strength of the aqueous medium

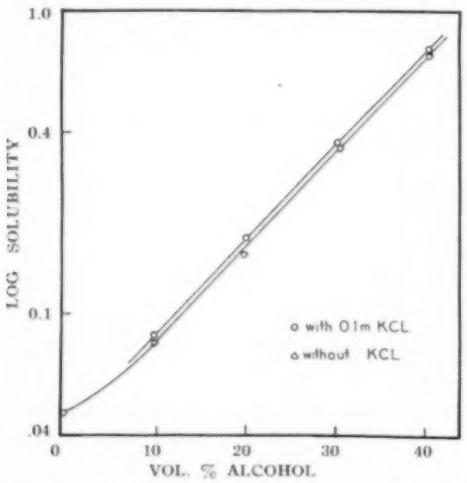

Fig. 6.—Influence of alcohol concentrations on the solubility of unionized sulfathiazole in the presence of KCl.

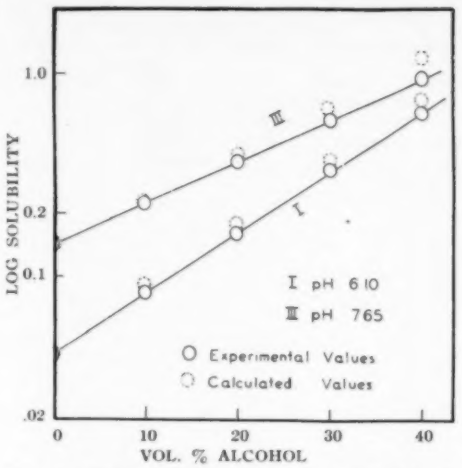

Fig. 7.—The solubility behavior of sulfathiazole in buffered solutions containing various percentages of alcohol.

seems generally to result in increased solubility. The slight increase in solubility for sulfathiazole is thus attributable to its somewhat polar character.

Figures 7 and 8 show the solubility behavior of sulfathiazole in buffered solutions containing various percentages of alcohols. The smooth curves in these plots represent the experimentally determined solubilities at the several hydrogen ion concentrations. The broken circles represent the values derived from the equation by substituting the S_o values from Fig. 6 and pK_a values at the alcohol concentrations shown.

The dissociation constants in alcohol were determined directly from potentiometric titration curves of sulfathiazole in hydroalcoholic solutions. The influence of alcohol in depressing the degree of ionization is shown in Fig. 9 where the apparent

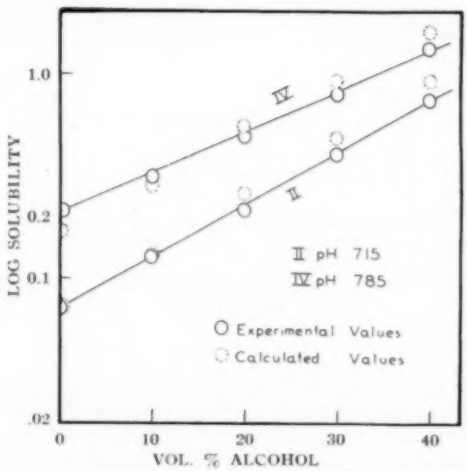

Fig. 8.—The solubility of sulfathiazole in buffered solutions containing various percentages of alcohol.

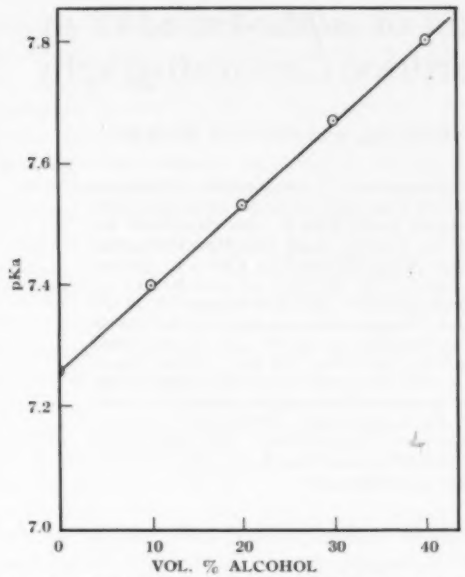

Fig. 9.—The influence of alcohol on the dissociation constant of sulfathiazole.

pK_a has been plotted against alcohol concentration. This curve is in excellent agreement with that of Stockton and Johnson (7). Although the pK_a values obtained in the presence of alcohol do not represent the true thermodynamic values (since the potentiometric titration curves based on glass electrodes readings are not true measures of thermodynamic *p*H values), the errors introduced are compensated by the fact that the apparent *p*H readings for the solubility determinations were also carried out in the presence of alcohol.

It is evident (Figs. 7 and 8) that Eq. 2 is sufficiently accurate for all practical purposes to predict the influence of both alcohol and *p*H on the solubility of sulfathiazole and other weakly acidic drugs which do not form interfering complexes. According to the equation, supported by experimental data, addition of alcohol to buffered systems generally influences the solubility of drugs such as sulfathiazole in two ways. In one sense, it decreases the solubility of the drug by reducing its dissociative tendency. On the other hand, it increases the solubility of the unionized form by decreasing the polarity of the solvent. For nearly all substances, the latter effect would probably mask the former, leading to a net increase in solubility.

Equation 2 permits close estimation from a very limited amount of data of the solubility of most slightly soluble drugs in aqueous or fixed hydroalcoholic solutions as a function of *p*H. It is only necessary to know S_0, the solubility in an unbuffered medium, and a single solubility value in a buffered solution at a *p*H slightly above the pK_a of the drug. These two values permit drawing of the solubility function on a log $S - S_0$ *vs.* *p*H diagram, since a single point and the fixed slope of one defines the straight line function. For approximate solubilities at *p*H values well above the pK_a of the drug concerned, only a single solubility determination is necessary, since S_0 may be neglected in comparison with the over-all solubility.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal, and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Lauryl pyridinium iodide
Stigmasteryl acetate
Cholestanone
Thymidine
D-Galactoascorbic acid
Carbobenzoxy-L-isoglutamine
Lithocholic acid
Mellitic acid
ortho-Aminophenylarsonic acid
2,6-Lutidine
Allyl propyl disulfide
1,2,3,4-Tetracarboxybutane
Lignoceryl alcohol
Eicosanol
Arachidic acid
Androsterone
Myoglobulin
Ergothioneine
Ellagic acid
Ficin

Colorimetric Determination of *alpha*-Estradiol in Estrogenic Mixtures Using Partition Chromatography*,†

By EDWARD O. HAENNI,‡ JONAS CAROL, and DANIEL BANES§

The use of iron-Kober reagent for the determination of α-estradiol in estrogen preparations has been limited by the interfering reactions of some other phenolic estrogens. A simple partition chromatographic procedure is now described for separating the estradiols and dihydroequilin as a group from complex estrogenic mixtures. The partition system comprises 0.4 *N* NaOH fixed on Celite as the immobile phase with benzene as the mobile phase. By dilution of iron-Kober reagent with 1 *N* HCl, a new reagent for the determination of α-dihydroequilin in the presence of the estradiols has been prepared. Results of analysis of mixtures of α- and β-estradiols with α-dihydroequilin containing up to 40 μg. of estrogens in various proportions show good precision and accuracy for each of the estrogens by the combined iron-Kober procedures. By using previously described methods for the extraction and separation of the ketosteroids in combination with the new methods, an analytical procedure for the colorimetric determination of α- and β-estradiols in complex mixtures of estrogens is presented. The results obtained by six laboratories in the analysis of two oil solutions of such complex mixtures show that the procedure is satisfactory.

AN EARLIER report (1) on a sensitive iron-Kober reagent for the determination of α-estradiol in the presence of β-estradiol described its application to the analysis of pharmaceutical preparations essentially free of other phenolic estrogens. Many commercial estrogenic preparations are complex mixtures of ketosteroids and their reduction products, the diols. Methods have been developed in this laboratory for the gravimetric determination of the total ketosteroids (2) and for their individual determination by colorimetry (3) and infrared spectrophotometry (4). One of the authors (5) has also published a method for the infrared spectrophotometric assay of the estrogenic diols, incorporating for some mixtures the partition chromatographic system described herein for the separation of the dihydroequilenins from α-dihydroequilin and the estradiols. The unique character of the infrared absorption spectra of such compounds makes this method of particular value. However, the limited availability of infrared spectrophotometers and the need for more sensitive methods in some cases made it advisable to extend the colorimetric procedures to the assay of estrogenic mixtures. α-Estradiol in particular must be determined in such mixtures because of its high estrogenic potency and because the proportion present may serve as an indication of sophistication in products claimed to be mixed estrogens from natural sources. Accordingly the primary object of the method herein described is the determination of α-estradiol in estrogenic mixtures, although the concurrent determination of α-dihydroequilin and β-estradiol is also involved.

The procedure described by Carol and Rotondaro (2) using the well known Girard reagent T for the isolation of the ketosteroids permits only traces of ketosteroids to pass into the nonketonic extract, so that the ketosteroids are a minor source of interference in such extracts. However, the nonketonic extract may contain α- and β-dihydroequilenins, α-dihydroequilin,[1] and the estradiols, in addition to solvent residues and weakly phenolic impurities. As indicated in a previous report (1) the dihydroequilenins and dihydroequilin react with iron-Kober reagent to some extent. Moreover, excessive nonestrogenic impurities potentially present in the nonketonic extracts from crude mixed estrogens may yield so much nonspecific color because of charring during the iron-Kober reaction that the accuracy of the correction provided for such interference is seriously affected. A preliminary study of the partition coefficients of β-dihydroequilenin and β-estradiol between organic solvents and buffers in the high *p*H range indicated the possibility of

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† The steric designations used conform to those in the U. S. Pharmaceopeia and in earlier reports in THIS JOURNAL.

‡ Bureau of Entomology & Plant Quarantine, U. S. Department of Agriculture, Beltsville, Md.

§ We wish to express thanks for cooperation in the collaborative analyses to Dr. L. I. Pugsley, Chief, Food and Drug Division, Department of National Health and Welfare, Ottawa, Canada; and to H. Isacoff, H. O'Keefe, A. W. Steers, and H. Van Dame, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] The authors reported the existence of β-dihydroequilin (6), a diol unknown when the present work was undertaken, probably because of its destruction in the usual acid hydrolysis procedures for isolating hormones from pregnant mare's urine. For the same reason it is not likely to be found in appreciable proportions in pharmaceutical estrogenic preparations. Its net effect on the α-estradiol determination is less than that of α-dihydroequilin but is still significant. It appeared in the estradiol chromatographic fraction of this method and could be detected by the test previously reported (6). Its separation from the estradiols is possible either by acid isomerization of β-dihydroequilin to Compound 3 (7) and application of the partition system of the present procedure or by direct use of a sufficiently long partition column as suggested in the following paper (8).

readily separating the dihydroequilenins from the estradiols by partition chromatography. Taking advantage of the fact that alkali solutions are themselves buffers at higher concentrations and finding that Celite was a suitable carrier for the alkali, a partition column was developed consisting of 0.400 *N* NaOH as the nonmobile solvent on Celite No. 545 and benzene as the mobile solvent. A short column permits ready separation of the dihydroequilenins from the dihydroequilin and the estradiols. At the same time it serves to eliminate the bulk of the nonestrogenic impurities and possible traces of estrone and equilin in the fore-run immediately preceding the eluate fraction containing the dihydroequilins and estradiols.

A study of the reaction of α-dihydroequilin with iron-Kober reagent resulted in the development of a new reagent (Reagent C) for this diol made by dilution of the stock iron-Kober reagent (Reagent A) with normal hydrochloric acid. Reagent C produces an intense yellow color with α-dihydroequilin on short standing at room temperature. This color is an intensification of that produced by Reagent B (Fig. 1). The hydrochloric acid has a stabilizing effect and slightly shifts the maximum absorption to 472 mμ. The optical density at 472 mμ of the color produced is proportional to the α-dihydroequilin concentration within practical limits even in the presence of relatively large proportions of the estradiols (Fig. 2). The determination of α-estradiol in the dihydroequilin-estradiol fraction is therefore based on the application of procedures A and B of the previously published estradiol method (1) and of procedure C for the estimation of α-dihydroequilin.

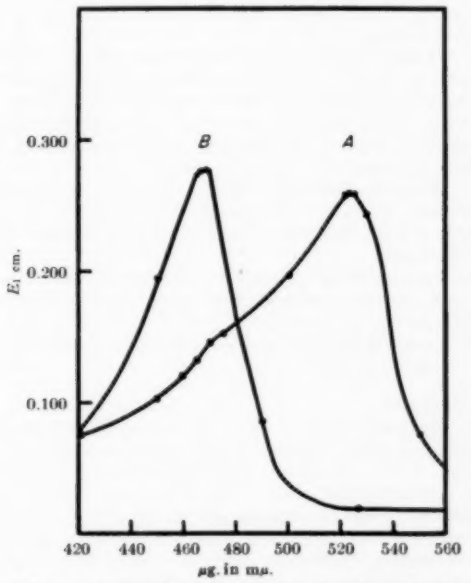

Fig. 1.—Absorption spectra of colors formed with 20 μg. of α-dihydroequilin per 5 ml. in Procedures A and B.

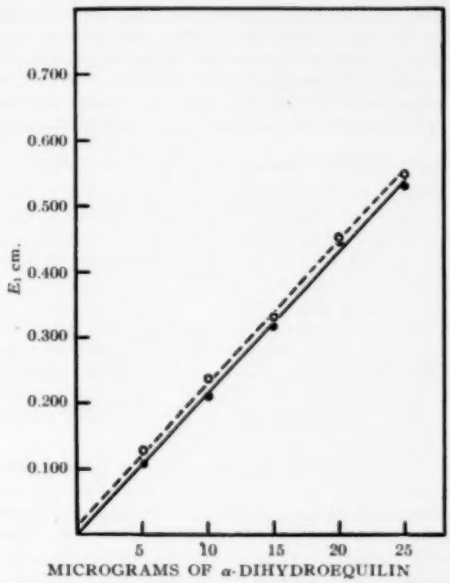

Fig. 2.—Optical density at 472 mμ *vs.* concentration (μg./5 ml.) in Procedure C for α-dihydroequilin (●) and α-dihydroequilin plus 25 μg. of α-estradiol and 25 μg. of β-estradiol (○) (single determinations).

EXPERIMENTAL

Partition Column.—A general report on the partition chromatography of the estrogenic diols with columns of the type used in this work is given in another paper (8). Accordingly, only a limited background is presented here bearing on a specific application of the short column. For the present work, the primary function of the partition column is to separate the α-estradiol from the dihydroequilenins. Hence the procedure adopted was designed to include all of the estradiol fraction. In a typical short column, as described in this procedure, the threshold values (including the benzene used to transfer the estrogens to the column) for β-estradiol,[2] α-estradiol, α-dihydroequilin, and β-dihydroequilenin are 70 ml., 80 ml., 130 ml., and 310 ml., respectively. The corresponding cut-off values are 140 ml., 150 ml., 240 ml., and 480 ml. The method provides for a cut-off volume up to 220 ml., depending on the temperature. This insures complete removal of the estradiols from the column free of dihydroequilenins. The estradiol fraction may not contain all of the α-dihydroequilin under some conditions. The rejected fore-run contains most of the nonestrogenic im-

[2] The 17 β-epimer precedes the 17 α-epimer off the column for all pairs studied.

purities and the possible traces of ketosteroids in the extract. In the authors' experience with the column there is no evident variation of practical significance in the threshold values with several lots of Celite No. 545 obtained at different times.

Interfering Color Reactions of α-Dihydroequilin in Procedures A and B.—The absorption spectra of the colors formed with α-dihydroequilin in procedures A and B are shown in Fig. 1. In procedure A, α-dihydroequilin produces a red color with a maximum absorption peak at 524 mμ, the same as that observed with α-estradiol, but of about one-half the intensity. Moreover, the absorption at 420 mμ is considerably higher in the case of α-dihydroequilin, so that its effective concentration using the optical density relation, $E_{525\ m\mu} - \frac{E_{420\ m\mu}}{2}$, is only about two-fifths that of α-estradiol. The optical density relation *vs.* concentration deviates somewhat from a straight-line function. Thus, the presence of 5, 10, 15, and 20 μg. of α-dihydroequilin may appear as 1.8, 3.9, 6.1, and 8.7 μg., respectively, of α-estradiol in procedure A. For the quantities of dihydroequilin usually encountered in this procedure (below 10 μg.) the error in the apparent α-estradiol effect resulting from ignoring the deviation and using a 10-μg. standard is negligible. At higher concentrations, correspondingly higher standards should be used to determine the correction.

The yellow color obtained with α-dihydroequilin in procedure B is a satisfactory qualitative index of the presence of a significant quantity of the estrogen in the presence of β-estradiol. Although it is not stable enough to yield an adequate two-color procedure, it is used for a preliminary screening measurement to detect the presence of a significant quantity of α-dihydroequilin. The absorption by the yellow color at 526 mμ causes little interference in the β-estradiol determination, but at high levels, the absorption at 420 mμ may introduce a significant error in applying the optical density relation prescribed. A standard of 10 μg. is adequate in procedure B for the quantities of α-dihydroequilin that will ordinarily be encountered. Beyond this limit, the error will increase at a progressively higher rate unless appropriate standards are used.

Color Reaction for the Determination of α-Dihydroequilin (Procedure C).—Reagent C, a mixture of reagent A and normal hydrochloric acid, is not as stable as desired. However, its ease of preparation from stock iron-Kober reagent makes this factor of less importance. The normality of the hydrochloric acid is not at all critical, but much higher concentrations cause increasing interference from the estradiols, while much lower concentrations decrease the stability of the color. (Although the use of a dilute solution of sodium metabisulfite in place of the hydrochloric acid produces a somewhat more intense and stable color, the resulting reagent is very obnoxious to use.) The time of attainment and the duration of the maximum yellow color tend to decrease with increase in the temperature and the age of the reagent. The intensity of the color is likewise affected, with the $E^{1\%}_{1\ cm.}$ value at 472 mμ ranging from 950 to 1,200. However, as shown in Fig. 3, the rate of development and the duration of the maximum are independent of the concentration within practical limits in the range of concentrations prescribed in the procedure. It is also evident that the color is sufficiently stable to permit satisfactory measurement of the maximum.

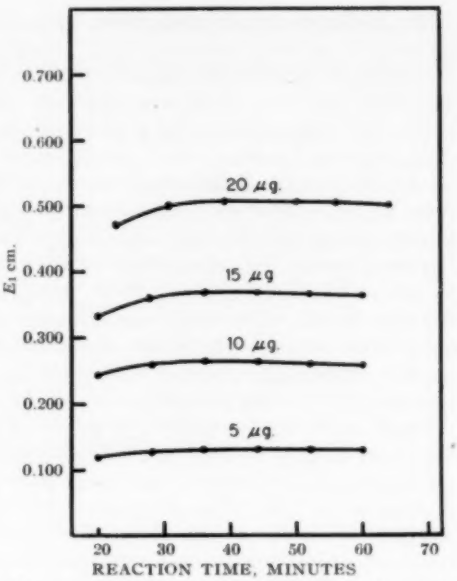

Fig. 3.—Color development-reaction time relation for various α-dihydroequilin concentrations (μg./5 ml.) with reagent C at 28°.

METHOD

The following items and directions are additions to or modifications of the procedures described in preceding papers (1, 2).

Apparatus

Partition Tube.—Select a 25 x 200-mm. test tube of 3.85 to 4.00 sq. cm. cross-sectional area by measuring the height of a 50-ml. column of H_2O in it. Fuse a 6-cm. length of 5 to 6-mm. tubing to an outlet in the bottom of the tube and slightly constrict this stem about 2 cm. below the joint.

Benzene Reservoir.—Use a 500-ml. separatory funnel with a 3-mm. or larger bore stopcock lubricated only with H_2O. The stem should be about 10 cm. long.

Packing Rod.—Flatten the end of a glass rod to a circular head with a clearance of about 1 mm. in the partition tube.

Leveling Rod.—Use a sharp-edged rod about 1.5 cm. in diameter.

Reagents

Reagent C.—To a carefully measured volume of reagent A in a glass-stoppered graduated cylinder, add 0.45 volumes of 1 *N* hydrochloric acid, mix vigorously, and place at once in a water bath at 25–28°. Use the reagent preferably after one hour and not more than three hours after its preparation.

Packing Material.—Celite No. 545 (Johns-Manville diatomaceous earth) is employed.

Sodium Hydroxide Solution.—The sodium hydroxide solution used is 0.400 *N* (carbonate-free).

Standard Solution

α-Dihydroequilin Standard.—Prepare a solution of 10 μg./ml. in the same way as the estradiol standards from 5 mg. of pure α-dihydroequilin. Store in the dark.

Sample Solution

Transfer a portion of the oil (or the estrogenic extract from tablets or aqueous suspensions, dissolved in 5 ml. of ether or chloroform) containing 0.2 to 0.5 mg. of estradiol or 50,000–100,000 I. U. of mixed estrogens to a 125-ml. separatory funnel with 25 ml. of Skellysolve C. Proceed by the method of Carol and Rotondaro (2) to the point where the solution obtained in the Girard reaction has been neutralized. Extract at once with three successive 15-ml. portions of chloroform. Drain each portion of chloroform successively into a second separatory funnel containing 5 ml. of water, wash, and filter through a pledget of cotton wet with chloroform into a 50-ml. glass-stoppered volumetric flask. Dilute to 50 ml. with alcohol. Take care at all times to avoid unnecessary heating of dry estrogenic extracts.

Preliminary Determination

Apply procedures A and B below to aliquots of the sample solution, disregarding the presence of any α-dihydroequilin. (Very turbid solutions sometimes result from these direct procedures. Filter such solutions, as well as the solutions obtained with the blanks and standards, through a pledget of fine glass wool packed tightly in the lower end of the stem of a Bunsen funnel.) The apparent α-estradiol so determined is the maximum quantity present and serves as an indication of the need for further assay.

Determination

Preparation of Partition Column.—Pack fine glass wool into the constricted stem of the partition tube so that when the tube is filled with benzene, the rate of flow is within 2.5 to 3.0 ml./min. Before packing the column, fasten a piece of rubber tubing with attached screw-clamp to the outlet to limit the flow during packing. To 1 Gm. of Celite in a small beaker, add sufficient benzene to cover the Celite. With a pipette, add 0.5 ml. of H_2O and mix vigorously with a stirring rod until the Celite is uniformly wet. When the tube is about one-quarter filled with benzene, place a pad of glass wool (approximately 1 cm. high when gently compressed) at the bottom of the tube and then transfer the Celite mixture to the tube. Form a flocculent suspension by slowly working the packing rod up and down as a piston through the Celite mixture. Gently compress the Celite with the packing rod and finish off the edges with the leveling rod to form a level, sharply defined surface on a uniform pack approximately 1 cm. high. Cover 8 Gm. of Celite in a mortar with about 40 ml. of benzene and distribute exactly 5 ml. of 0.400 *N* NaOH over the Celite with a pipette. Mix carefully for several minutes with a pestle until the Celite appears uniformly wet. Open the screw-clamp enough to permit slow drainage during packing of the tube. Transfer the Celite to the tube with a spatula in about five portions, suspending each portion and gently packing as above.

Finish off the top of the column, scraping down any Celite on the upper wall of the tube to form a sharply defined level surface on a column of approximately 30-cc. volume over the initial pack. (Packing too tightly may cause loss of estradiol in the forerun, particularly at room temperatures much above 25°.) The Celite must be covered with benzene at all times. Nearly fill the separatory funnel with benzene and seal the stopper with a film of H_2O to prevent air leaks. Insert the stem of the funnel into the benzene over the Celite, fully open the stopcock, and adjust the level of the benzene to produce a flow-rate of 2.0 to 2.5 ml. per minute with the screw-clamp fully open. Mark the level of the benzene on the tube, close the stopcock and remove the benzene reservoir.

Partition.—Carefully evaporate to just short of dryness an aliquot of the $CHCl_3$ extract containing 100–250 μg. of total diols calculated as α-estradiol. If necessary, add a few ml. of alcohol near the end of the evaporation to aid in removal of any residual H_2O. Remove the last portions of solvent in an efficient desiccator connected to the vacuum for one hour or more. Dissolve the dry extract in 5 ml. of benzene by warming gently, then cool the solution to room temperature or below. Remove the rubber tubing from the partition tube, and as soon as the benzene stops dropping from the tube, transfer the diol solution to the tube at once with a 5-ml. pipette, allowing it to flow down the wall near the top of the Celite. When the benzene just stops dropping from the tube, complete the transfer in like manner with three more 5-ml. portions of benzene, discarding the effluent. Immediately place a 50-ml. graduated cylinder under the tube, add benzene to the level marked on the tube, and replace the benzene reservoir, supporting it at a height to maintain that level when the stopcock is fully opened. When exactly 30 ml. of effluent collects in the cylinder, replace the cylinder as receiver with a dry 250-ml. beaker previously marked at the 170-ml. level. (Decrease the 30 ml. of forerun by 2 ml. for each degree that room temperature exceeds 25°.) Collect 170 ml. of effluent in the beaker, concentrate the solution to 30–40 ml., transfer to a 50-ml. volumetric flask, and make to volume.

Determine α-estradiol in the solution by Procedures A, B, and, if necessary, C.[3]

Procedure A.—Proceed as directed (1) using as an additional standard, if necessary, 1 ml. of α-dihydroequilin standard.[4] Calculate the quantity in μg. of total estradiols (T_a) and of β-estradiol (B_a) in the aliquot by the following formulas:

$$\text{Micrograms of } \alpha\text{-dihydroequilin (DHQ) (calculated as } \alpha\text{-estradiol) in aliquot} = \frac{\text{Micrograms DHQ in aliquot (from Procedure C)}}{\text{Micrograms DHQ standard}} \times$$

$$\frac{20[E_{525\ m\mu}\ \text{DHQ standard} - (E_{420\ m\mu}\ \text{DHQ standard}/2)]}{E_{525\ m\mu}\ \alpha\text{-estradiol standard} - (E_{420\ m\mu}\ \alpha\text{-estradiol standard}/2)} = D_a$$

$$\text{Micrograms of } \alpha\text{-estradiol in aliquot} = T_a - B_a - D_a$$

Procedure B.—Proceed as directed (1) using 1 ml. of α-dihydroequilin standard 4 as an additional

[3] See footnote 1.

[4] Use of this 10-μg. standard for up to 15 μg. of α-dihydroequilin will not ordinarily cause errors of practical consequence. Beyond this limit, appropriate higher standards should be used.

standard. Measure the optical densities promptly at 468 mμ (maximum for α-dihydroequilin), at 420 mμ, and at 526 mμ. If $E_{468\ m\mu}$ sample − ($E_{526\ m\mu}$ sample × $E_{468\ m\mu}$ β-estradiol standard/$E_{526\ m\mu}$ β-estradiol standard) does not exceed 20% of $E_{468\ m\mu}$ α-dihydroequilin standard, then disregarding the presence of α-dihydroequilin in a like aliquot will result in not more than 0.8 μg. of apparent α-estradiol in Procedure A. If, as usual, this is a negligible quantity, calculate α-estradiol as follows and omit the dihydroequilin standard (Procedure A):

$$\text{Micrograms of } \beta\text{-estradiol in aliquot} = 20 \times \frac{E_{526\ m\mu}\text{ sample} - (E_{420\ m\mu}\text{ sample}/2)}{E_{526\ m\mu}\ \beta\text{-estradiol standard} - (E_{420\ m\mu}\ \beta\text{-estradiol standard}/2)}$$

Otherwise determine α-dihydroequilin by Procedure C and calculate β-estradiol as follows, where DHQ = α-dihydroequilin:

$E_{526\ m\mu}$ sample (corrected) = $E_{526\ m\mu}$ sample − ($E_{526\ m\mu}$ DHQ standard × μg. DHQ in aliquot/10)

$E_{420\ m\mu}$ sample (corrected) = $E_{420\ m\mu}$ sample − ($E_{420\ m\mu}$ DHQ standard × μg. DHQ in aliquot/10)

$$\text{Micrograms of } \beta\text{-estradiol in aliquot} = 20 \times \frac{E_{526\ m\mu}\text{ sample (corrected)} - [E_{420\ m\mu}\text{ sample (corrected)}/2]}{E_{526\ m\mu}\ \beta\text{-estradiol standard} - (E_{420\ m\mu}\ \beta\text{-estradiol standard}/2)}$$

Procedure C.—Transfer in duplicate to 18 x 150-mm. test tubes (1) an aliquot of the sample solution containing up to 20 μg. of α-dihydroequilin and 2 ml. of α-dihydroequilin standard (2). Add several pieces of silicon carbide to each tube, evaporate the solvent, and dry the residue exactly as described in Procedure A. Place the tubes in a water bath at 25–28° and add 5 ml. of reagent C rapidly near the bottom of each. Using a long stirring rod, mix the residue vigorously with the reagent for at least one minute. Leave the rod in the tube. Measure the optical densities of the sample and standard relative to the reagent (also held in the bath at 25–28°) at 472 mμ just thirty minutes after addition of the reagent and repeat the measurement at five to ten-minute intervals until the maximum optical density is reached (usually thirty-five to fifty-five minutes after mixing).

$$\text{Micrograms of } \alpha\text{-dihydroequilin in aliquot} = 20 \times \frac{E_{472\ m\mu}\text{ sample}}{E_{472\ m\mu}\text{ standard}}$$

DISCUSSION

The precision and accuracy attainable with the colorimetric procedures in the assay of mixtures of α-estradiol, β-estradiol, and α-dihydroequilin are shown in Table I. The complete procedure was submitted to collaborative study in six laboratories.[5] For this purpose, two samples of estrogenic mixtures in sesame oil were prepared. Their compositions were not revealed to the collaborators. The results reported for α-estradiol and β-estradiol are shown in Table II. Because the partition procedure was not designed to insure complete recovery of α-dihydroequilin, the collaborators were not requested to report α-dihydroequilin. The lack of a specific reaction for α-estradiol makes necessary the rather involved isolation procedure and the estimation of α-estradiol by difference, both factors increasing the chance of error. The collaborative test was severe, particularly in the case of Sample I. In view of the minute quantities of α-estradiol determined in the presence of relatively large quantities of interfering substances and of the limited experience of some of the collaborators with such procedures, the results reported are quite satisfactory.

SUMMARY

1. A colorimetric method has been described for the estimation of α-dihydroequilin in the presence of α- and β-estradiols. The reagent used is prepared by dilution of stock iron-Kober reagent.

2. A simple partition chromatographic method has been developed for separating α- and β-estradiols and α-dihydroequilin from the dihydroequilenins. The partition system consists of 0.4 *N* sodium hydroxide as immobile solvent fixed on Celite and benzene as the mobile solvent.

3. These methods, combined with previously described procedures, have been applied to the determination of α- and β-estradiols in estrogenic mixtures including the ketosteroids.

[5] The details of this study were previously reported (9).

TABLE I.—ANALYSIS OF MIXTURES OF α-DIHYDROEQUILIN, α-ESTRADIOL, AND β-ESTRADIOL

Sample No.	α-Dihydroequilin Present, μg.	α-Dihydroequilin Found, μg.	α-Dihydroequilin Av. Recovery, %	β-Estradiol Present, μg.	β-Estradiol Found, μg.	β-Estradiol Av. Recovery, %	α-Estradiol Present, μg.	α-Estradiol Found, μg.	α-Estradiol Av. Recovery, %
1	30.0	29.7	97.5	10.0	10.1	101.5	0.0	0.0	...
		28.8			10.2			0.0	
2	30.0	30.2	102	5.0	5.2	103	5.0	4.5	92
		30.9			5.1			4.7	
3	20.0	19.7	98.5	5.0	5.1	103	10.0	9.1	95.5
		19.7			5.2			9.9	
4	10.0	9.4	96	10.0	10.1	101	10.0	9.5	100
		9.8			10.1			10.5	
5	5.0	5.2	106	15.0	14.0	97	5.0	4.7	103
		5.4			14.7			5.6	
					14.9				
					14.7				
6	10.0	9.9	97	0.0	0.1	...	20.0	20.2	102
		9.5			0.2			20.6	

TABLE II—COLLABORATIVE ANALYSIS OF ESTROGENIC MIXTURES IN OIL

Analyst	Sample I[a] α-Estradiol, μg./Ml.	Sample I[a] β-Estradiol, μg./Ml.	Sample II[b] α-Estradiol, μg./Ml.	Sample II[b] β-Estradiol, μg./Ml.
1	23	60	30	6
2	23	67	24	9
3	22.5	59	28.5	6.5
	22.5	59.5	27	6
4	21	60	31	6
5	18	59	30	6
6	19	51	28	6
Found (Av.)	21	59	28	6.5
Present	20	61	30	6

[a] Sesame oil solution containing in addition to the estradiols: estrone, 0.79 mg./ml.; equilin, 0.31 mg./ml.; equilenin, 0.25 mg./ml.; α-dihydroequilin, 30 μg./ml.; α-dihydroequilenin, 50 μg./ml.; β-dihydroequilenin, 50 μg./ml.
[b] Sesame oil solution containing in addition to the estradiols: estrone, 0.50 mg./ml.; equilin, 0.10 mg./ml.; equilenin 0.05 mg./ml.

4. Collaborative tests of the methods in six laboratories on two estrogenic mixtures in oil indicate that the methods are satisfactory for the determination of α- and β-estradiols in complex mixtures containing the estrogenic ketosteroids as well as other estrogenic diols.

5. Although the acid-labile β-dihydroequilin is unlikely to be encountered in pharmaceutical preparations, the presence of material proportions would yield high results for the estradiols in the present procedures. Means of detecting and eliminating β-dihydroequilin are suggested.

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Partition Chromatography of Some Estrogenic Diols*,†

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A partition chromatographic system, comprising NaOH solution fixed on Celite as the immobile phase and benzene as the mobile phase in the separation of estrogenic diols, and various factors affecting the system are presented in detail. Procedures are described using a short column to permit ready separation of the estradiols and dihydroequilins as a group from the dihydroequilenins and a longer column to separate the estradiols from the dihydroequilins. Partition coefficients have been determined for β-estradiol and α-dihydroequilin over the range 20–35°. By the application of the Martin and Synge theory to the behavior of these estrogens on the column, it has been found that observed elution volumes agree reasonably well with values expected from the partition coefficients. Threshold and cut-off volumes for the estrogens are linear functions of the total volume required to remove the zone of maximum concentration from the column. Using these relations, the limits of separation of estrogens attainable by this system can be estimated from their partition coefficients or their behavior on a column. Relative partition coefficients determined from the columns are given for α- and β-estradiols, α- and β-dihydroequilins, and β-dihydroequilenin.

IN THE COURSE of the development of colorimetric methods (1, 2) for the determination of the various estrogenic diols present or potentially present in preparations derived from equine pregnancy urine, the lack of specificity of such methods made it necessary to find practical means of separating the diols. Initially promising results with Stimmel's chromatographic adsorption technique (3) led to the disappointing discovery that different lots of activated alumina showed persistent variation in activity. The impressive success in the separation of the fatty acids by partition chromatography (4–6) and the known differences in acidity of some estrogens suggested the possibility of using the newer technique for the separation of the estrogenic diols. The simple, clean-cut separation of the estradiols

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† The steric designations used conform to those in the U. S. Pharmacopeia and in earlier reports in THIS JOURNAL.
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and the dihydroequilenins which was immediately achieved led to the detailed study of the applicability of the method to further estrogen separations. Previous publications from this laboratory have described the application of this method to the quantitative analysis of estrogenic mixtures (7) and to the preparation, isolation, and identification of new estrogenic compounds (8, 9). However, the development of the method, some general principles involved, and some details of procedures have not been described heretofore.

Although, as indicated above, the usefulness of the new procedure for research purposes was recognized, the primary objective of this investigation was the development of an analytical method suitable for routine use in the examination of pharmaceutical preparations of estrogens. Because of the pharmaceutical significance of α-estradiol, the work was directed especially toward its determination. The empirical character of the chromatographic technique is particularly troublesome in dealing with colorless substances on a routine analytical basis. The minute quantities of the weakly phenolic estrogens encountered in this field made the use of acid-base indicators or fluorescence[1] impractical. Accordingly a detailed study of the variables affecting the operation of this partition system was undertaken to establish a standardized procedure.

EXPERIMENTAL

Partition System

Adsorbent.—The choice of adsorbent for the immobile phase was based on the ready availability of an inexpensive, at least partially standardized product, with high absorptive capacity, good flow characteristics, and relative inertness to strong alkali. Celite[2] No. 545, which had been used in partition chromatography (6), was found quite suitable for the present purpose. Three separate lots of Celite No. 545 purchased at intervals over a period of at least five years showed identical threshold values for α-estradiol when tested in a standard short column prepared with 5 ml. of alkali solution to 8 Gm. of Celite. The ratio of 20 ml. of alkali solution to 21 Gm. of Celite used in the long column is slightly less than the maximum proportion of aqueous phase found to give a pack with satisfactory physical characteristics.

Immobile Phase.—A considerable amount of exploratory work involving distribution experiments as well as partition chromatograms preceded adoption of 0.4 N NaOH as the immobile phase for this procedure. In this work the concentration of alkali ranged from 0.05 N to 1.0 N. Obviously there is a wide latitude of choice. Preliminary approximate partition coefficient determinations, for example, indicated that a column made up with 1.0 N NaOH to produce a given separation of β-estradiol from α-dihydroequilin would have to be about one and one-half times as long when using 0.4 N NaOH or about seven times as long when using 0.05 N NaOH to produce the same separation, with all other conditions the same. The choice of 0.4 N concentration was based on the use of a minimum quantity of developing solvent for a column of suitable dimensions to effect the particular separations desired. Also, the 0.4 N NaOH is sufficiently concentrated to be in the highly buffered range (10). In several chromatograms employing 1.0 N NaOH, a reddish residue of undetermined origin found in the estradiol fractions eluted from the column interfered with the estradiol determination. Although the limited experiments with chromatograms, using concentrations other than 0.4 N NaOH, do not permit definite conclusions on the matter, the data suggest that the width of the developed band increases with NaOH concentration, thus decreasing the efficiency of separations.

Mobile Phase.—Preliminary distribution experiments with the solvents n-hexane, cyclohexane, carbon tetrachloride, chloroform, and benzene indicated that only the last two would be suitable for the partition of the estrogenic diols on alkaline columns. Benzene was preferred primarily because the differences in partition coefficients for the estradiols and α-dihydroequilin are several times greater in benzene than they are in chloroform. Moreover, the very low solubility of the alkali in benzene makes it unnecessary to saturate the benzene with alkali before use. The great density of chloroform also introduces some difficulties in the preparation of a column.

Partition Conditions

Flow-rate.—Martin and Synge (11) pointed out that there is an optimum flow-rate for a partition column in any given case. The factor of diffusibility of the solutes governs the importance of the flow-rate in those cases where uniformity of flow is not seriously impeded by the degree of packing. Cook (12) reported a slight increase in band volume when the flow-rate was reduced from 5 ml./min. to 50 ml./hr. and a rapid increase with further reduction in rate. On the other hand, Levi (13) found that too rapid a solvent flow gives diffuse columns. Obviously, each partition system must be studied individually with regard to this factor. In the present case, flow-rates were varied from 0.9 ml./min. to 4.0 ml./min. However, it was found that the factors of temperature, ratio of immobile to mobile phase, and degree of packing are so much more important that the effect of flow-rate within the range studied is difficult to establish with certainty. On the basis of the data available, flow-rates of 2.0 ml./min. to 3.0 ml./min. seem preferable. Because of the importance of standardizing conditions for the use of an empirical procedure in routine work, it is recommended that this range be adhered to in the analytical method. The device previously described (14) for controlling the flow-rate produces a gradual but limited increase in rate as the level of benzene in the reservoir recedes.

Temperature.—Strain and Murphy (15) called attention to the failure of many investigators to

[1] The fluorescence of some estrogens under ultraviolet light is useful in following certain macro separations, but it is too weak with usual analytical quantities.

[2] Johns-Manville diatomaceous earth.

mention important factors affecting reported R_F values. Bate-Smith and Westall (16) have particularly emphasized the effect of temperature on R_F values. The present work was begun at a season when room temperature varied over a narrow range and the effects were obscured by the other factors under investigation. Anomalous results obtained during a period of high temperatures led to determination of the variation of the partition coefficients of β-estradiol and α-dihydroequilin with temperature, and showed that it is a most important factor in this system. The results are plotted in Fig. 1, together with the difference in partition coefficients at various temperatures which determines the degree of separation on a column. It will be noted that this difference varies by more than 100% within a temperature range that might normally be expected to occur during the year. The decrease in fore-run with increased temperature described in the analytical method for α-estradiol (14) is based on the indicated decrease in the partition coefficient of β-estradiol. Throughout this report the term partition coefficient refers to the ratio of the concentration of solute in the alkali phase to its concentration in the organic solvent phase. Partition coefficient determinations were made at concentrations comparable to those used in the analytical procedure. Because of the low concentrations used and the limitations of the colorimetric methods of determination, considerable difficulty was experienced, particularly for the higher partition coefficients. However, the reported values are based on complete accounting for the estrogen in both phases within the limits of experimental error.

Another effect of temperature is less important but still significant. As the temperature increases, the band volume decreases (Table I). Hence, for a given separation of the zones of maximum concentration of the two substances, the separation developed at the higher temperature will give a greater actual separation. For example, in two separations under the same conditions except temperature, that made at 20° showed a separation of maximum concentration zones of 130 ml., but the actual separation of the β-estradiol and α-dihydroequilin was only 40 ml.; whereas that made at 29° gave corresponding values of 75 ml. and 30 ml. Moreover, at 20° complete removal of the β-estradiol and the α-dihydroequilin required 130 ml. and 290 ml., respectively, while at 29° the corresponding volumes were only 90 ml. and 185 ml. The efficiency of separation is accordingly actually greater at higher temperatures. This is of special significance where larger quantities of estrogens are concerned, because their high temperature coefficients of solubility permit use of lower volumes of initial solvent and increase the capacity of the column at higher temperatures.

Volume of Packed Column.—As indicated by Martin and Synge (11), the most efficient column theoretically should be tightly packed, but since uniformity of flow must also be promoted, an intermediate degree of packing is more efficient in practice. This efficiency, as mentioned above, is related to band volume. A study of the relation of band volume to the degree of packing indicates that the ratio of the alkali volume to the benzene volume in the pack should not appreciably exceed 0.5 and should preferably be in the range 0.4 to 0.5. In this study, fifty-one observations of the band volumes for 100-μg. quantities of α-estradiol, β-estradiol, α-dihydroequilin, and β-dihydroequilenin on both short and long columns, using 0.4 N NaOH and benzene, showed minimum band volumes in the packing range recommended. As previously noted by Cook (12) for other partitions, a simple linear relation was found between the volume of developing solvent required to elute the maximum concentration of solute and the band volume. Thus, for the recommended range of packing, the nine observations made showed almost perfect correlation in the regression relation:

$$\text{Band volume (ml.)} = 18 + 0.38 \times \text{development volume (ml.)}$$

The coefficient of determination was 99% and the standard error of estimate was 6.3, adjusted for the small number of cases. Table I shows the average percentage deviation of all the observations in various packing ranges from this minimum band volume for both normal temperatures (below 27°)[1] and more elevated temperatures. Including all fifty-one observations, the range of development volumes was from 50 to 695 ml. and the range of band volumes was from 40 to 290 ml. For the nine observations in the regression calculation, the corresponding ranges were 75 to 615 ml. and 55 to 260 ml. respectively.

That the band volume for a fixed quantity of estrogen (and for a restricted initial solution volume) is primarily dependent on the volume of developing solvent is even more strikingly apparent from the fact that a linear relation derived from sixty-four observations, including variations in all known factors (flow-rate, degree of packing, ratio of alkali to benzene, temperature, and normality of alkali), showed a coefficient of determination of 87%.

In terms of the packing of the alkaline columns in which 5 ml. of NaOH to 8 Gm. (3.6 cc.) of Celite is used, the preferable range corresponds to the alkali-Celite volume constituting 41 to 46% of the total volume of the alkaline pack, excluding the volume of the water-Celite pre-pack. For columns in which 20 ml. of NaOH to 21 Gm. (9.5 cc.) of Celite is used, the preferable range corresponds to an alkali-Celite volume constituting 37 to 42.5% of the total volume.

The Short Column

The details of preparation of the short column have been published in connection with its use in the determination of α-estradiol (14, 17). In that procedure a somewhat looser pack was used than that recommended for the most efficient separation of estrogens. There is no danger of eluting any dihydroequilenin present under the prescribed conditions, and at the same time the looser pack precludes the chance of losing estradiol in the fore-run. However it was not the purpose to completely remove any α-dihydroequilin from the column, but only to assure complete recovery under all conditions of the α-estradiol which precedes it. The short column was also designed completely to separate the estradiols and dihydroequilin as a group from the dihydroequilenins. For this purpose, it is preferable to pack the column more tightly so that

[1] Many of the observations were made at uncontrolled room temperatures. Such observations made when outside temperatures exceeded 27° are included in the higher temperature category.

TABLE I.—EFFECT OF ALKALI/BENZENE RATIO AND TEMPERATURE ON BAND VOLUME

Range of Volume Ratios, 0.4 *N* NaOH/C_6H_6	Temperature <27° No. of Observations	Temperature <27° Av. % Dev. from Min. Band Vol.[a]	Temperature >27° No. of Observations	Temperature >27° Av. % Dev. from Min. Band Vol.[a]
0.20–0.29	9	+40	0	...
0.30–0.39	9	+17	9	+ 2
0.40–0.49	9	0	0	...
0.50–0.60	7	+33	4	+ 6
>0.69	0	...	4	+28

[a] Deviation from calculated band volume: band volume = 18 + 0.38 × development volume.

the alkali-Celite volume constitutes from 41 to 46% of the volume of the total alkaline pack. The tighter pack may decrease the threshold volume for the estrogens by as much as 10 ml. Moreover, because of the marked effect of temperature on the partition coefficients, it is necessary to make adjustments for both the threshold and the cut-off volumes.

Accordingly, pack the alkali-Celite to a volume of 19–21 ml. and proceed with the partition as previously described (14) to the point where the effluent is collected in a graduated cylinder. Instead of 30 ml., collect exactly 20 ml. (minus 2 ml. for each degree of room temperature above 25°). Change the receiver and collect 195 ml. *plus* 17 ml. for each degree of room temperature below 25° or *minus* 8 ml. for each degree above 25°. If the dihydroequilenins are to be determined, elute the column with 200 ml. of ether collected in another receiver. The benzene eluate and ether eluate[4] may then be analyzed in accordance with prescribed procedures (7, 14).

Use of the short column will ordinarily be adequate for most analytical purposes and is more economical of time and materials than the long column. However, in those cases where it is necessary to separate dihydroequilin from the estradiols, it is more efficient to proceed at once with the long column which permits separation of estradiol, dihydroequilin, and dihydroequilenin groups in one operation.

The Long Column

The technique of preparation of the long column is essentially the same as that of the short column. Make the partition tube by fusing a 6-cm. length of 5 to 6-mm. tubing to an approximately 25-cm. length of 25-mm. tubing of selected cross-sectional area as described for the short column. Slightly constrict the stem about 2 cm. below the junction. Pack the glass wool and the water-Celite pre-pack as for the short column. Cover 21 Gm. of Celite No. 545 in a mortar with benzene (80–100 ml.) and distribute exactly 20 ml. of 0.400 *N* NaOH over the Celite from a pipette. Mix carefully and thoroughly with a pestle until the Celite is uniformly wet. Prepare the alkaline pack in the same way as for the short column, transferring the Celite in about 10 portions and packing so that the volume of the alkali-Celite (excluding the pre-pack) is between 70 and 80 cc. Adjust the flow-rate up to 2.5 to 3.0 ml. per minute. Add the solution of diols in 5 ml. of benzene at room temperature to the column as completely as possible and transfer the three 5-ml. benzene washings to the tube as directed for the short column. Discard the effluent and place a graduated cylinder under the tube, adjusting the benzene reservoir to the proper level. Collect as fore-run 185 ml. of effluent plus 14 ml. for each degree of room temperature below 25° or minus 8 ml. for each degree above 25°. Change the receiver and collect 225 ml. plus 16 ml. for each degree of room temperature below 25° or minus 5 ml. for each degree above 25°. This is the estradiol fraction. Change the receiver and collect 320 ml. plus 24 ml. for each degree of room temperature below 25° or minus 15 ml. for each degree above 25°. This is the α-dihydroequilin fraction. Change the receiver and elute the dihydroequilenins with 250 ml. of ether.

DISCUSSION

The limiting values used in the method were deduced from observed relations between the threshold (T) and cut-off (C) volumes and the volume of benzene preceding the elution of the zone of maximum concentration (V) by the application of the Martin and Synge (11) theory of the partition chromatogram, which is based on the assumption that the partition system comprises a linear distribution isotherm. The justification for its use in the present instance, involving a distribution of ionic (and possibly associated) species between a buffer and an organic solvent, is first, that such a system may be expected to promote linearity in the effective distribution isotherm [Synge (18)]; secondly, that the theory developed for buffer systems [Levi (13)] reduces to the same equations for such dilute solutions as are considered here; and, finally, that V values calculated from the partition coefficients by the theory agree reasonably well with observed values. For example, if L is the volume of benzene in the alkali-Celite pack, v is the volume of alkali and k is the partition coefficient, then it is easily deduced from the Martin and Synge development that the volume of benzene required to move the zone of maximum concentration from the top to the bottom of the alkali-Celite pack is equal to: $L + vk$.

In the present procedure the pre-pack of water and Celite (used to wash traces of alkali from the benzene), together with the glass wool, holds approximately 5 ml. of benzene. Also it is reasonable to consider that for such dilute solutions, the initial 5 ml. of solution used to add the solute leaves the zone of maximum concentration at the top of the column. The total volume of benzene preceding the zone of maximum concentration in the eluate minus 10 ml. should equal the volume calculated by the equation. In Table II are shown the observed and calculated volumes for a series of chromatograms on short columns at several temperatures. The observed volumes are reported to the nearest 5 ml., the volume of the individual fractions collected.

[4] The dihydroequilenins may also be determined colorimetrically with iron-Kober reagent. Both α- and β-dihydroequilenins react in Procedure A of the estradiol method (2) but the colors have only about one-quarter of the intensity observed with α-estradiol. $E_{\lambda\ max.}$ is at 512 mμ for the α-dihydroequilenin color and at 516 mμ for the β-dihydroequilenin color. Optical density measurements at 514 mμ are directly proportional to dihydroequilenin concentrations up to at least 40 μg. In Procedure B, only β-dihydroequilenin yields a color ($E_{\lambda\ max.}$ at 516 mμ). This color intensity, however, is not a straight-line function of estrogen concentration; thus a standard curve must be prepared.

TABLE II.—OBSERVED AND CALCULATED ELUTION VOLUMES

	Vol. to Move Max. Concn. Zone Length of NaOH-Celite Column			
	β-Estradiol		α-Dihydroequilin	
Temperature, °C	Observed, Ml.	Calcd., Ml.	Observed, Ml.	Calcd., Ml.
20	75	92	205	207
25	70	66	160	148
27.5	65	55	...	...
27.5	60	58	...	...
27.5–28	70	59	...	...
29°	55	55	130	122

In general, the observed values show reasonable agreement with those calculated, although the values for β-estradiol at 20° are more divergent than expected.

The observed threshold (T) and cut-off (C) volumes relate to 100-μg. quantities of estrogen (α-estradiol, β-estradiol, and α-dihydroequilin) and are selected to include 99% of the estrogen. The relations between these volumes and the total volume (V) preceding the zone of maximum concentration from the long column are as follows:

$$T = 16 + 0.78\ V$$

$$n = 14;\ \bar{d} = 0.99;\ \overline{\text{S. E.}} = 7$$

$$C = 19 + 1.18\ V$$

$$n = 14;\ \bar{d} = 0.99;\ \overline{\text{S. E.}} = 9$$

Partition coefficients for α-estradiol were not determined. The cut-off values for α-estradiol at various temperatures were estimated from those for β-estradiol, using the observed relation:

$$V\ (\alpha\text{-estradiol}) = 13 + 1.11\ V\ (\beta\text{-estradiol})$$

$$n = 5;\ \bar{d} = 0.99;\ \overline{\text{S. E.}} = 7$$

The above observations include a range of V values from 190 ml. to 700 ml., corresponding to room temperatures ranging from approximately 22 to 35°. It must be emphasized that the column used in the procedure was designed primarily for estrogen capacities up to a few hundred μg. and there is likelihood of serious overlapping of zones with high loads and elevated temperatures. However, it has been used with satisfactory results (7) for quantities of estrogens up to 2.5 mg. at or below normal room temperatures (approximately 25°).

The preceding equations may be used (within the range of observations) to estimate the degree of separation (99%) between two estrogens to be expected on a given column if their partition coefficients are known or if their V values are known for a column of different size at the same temperature. Thus, if two estrogens are represented by the subscripts 1 and 2, and $k_2 > k_1$, the degree of separation is given by the relation:

$$T_2 - C_1 = 0.78\ V_2 - 1.18\ V_1 - 3\ \text{S. E.} = 16^{5}$$

As calculated from the k values for β-estradiol and α-dihydroequilin (Fig. 1), the separation between 100-μg. quantities of these estrogens on the standard long column at 25° should be 143–148 ml., depending on the degree of packing. The observed separations on three runs at room temperature (approximately 25°) ranged from 140 to 160 ml. Also, in a similar run on the short column at 25° (controlled temperature), the values for V_2 and V_1 were found to be 170 ml. and 80 ml., respectively, where L = 12 ml. From theory, $V_2 - V_1 = vk_2 - vk_1 = v\Delta k$. Hence, $\Delta k = \frac{V_2 - V_1}{v} = \frac{90}{5} = 18$. From these data it was calculated that the separation to be expected in a run on the long column when Δk was found to be 18 should have been 158 ml. for the L value of 58 ml. The separation observed was 140 ml. (probably due to somewhat loose packing of the column).

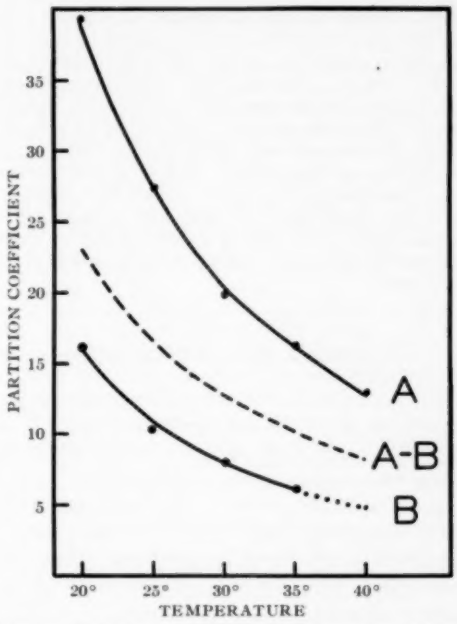

Fig. 1.—Partition coefficients *vs.* temperature. Curve *A*—α-dihydroequilin; Curve *B*—β-estradiol.

The above equations also permit estimation of the limit of the relation between partition coefficients of two estrogens, if they can be separated (99%) in a single run on a column of any length by the prescribed procedure. If H = the height of the alkali-Celite column, A_L = the area of the benzene phase and A_s = the area of the alkali phase in the cross section of the column, then

$$L = HA_L = HA_s\left(\frac{A_L}{A_s}\right) = v\left(\frac{A_L}{A_s}\right)$$

Hence $T_2 - C_1 = f(v)$ and, for the most tightly packed column recommended,

$$\left(\frac{A_L}{A_s} = 2\right),\ \frac{d(T_2 - C_1)}{dv} = 0.78k_2 - 1.18k_1 - 0\,8.$$

[5] The error is taken as the sum of the standard errors for the threshold and cut-off volume relations, because those conditions which contribute to a positive deviation in the one will cause a negative deviation in the other.

It is necessary for this derivative to be greater than zero if increasing the length of the column (v) is to produce an increase in separation (99%). It follows that $k_2 > 1.5\ k_1 + 1$ is the limiting relation sought. The k values at approximately 25°, as determined on columns, for a number of estrogenic diols are as follows: β-estradiol, 11; α-estradiol, 13; β-dihydroequilin, 20; α-dihydroequilin, 28; β-dihydroequilenin, 66. Thus it is obvious that one should not expect the following pairs to be separated (99%) by a single run in this system on a column of any length: α- and β-estradiols, α-estradiol and β-dihydroequilin, and α- and β-dihydroequilins. The relation between the values for α-estradiol and β-dihydroequilin is so close to the limiting value, however, that under most favorable conditions the separation might be attained on the standard long column by very close fractionation. It can be attained certainly by re-running the α-estradiol fraction containing a small amount of β-dihydroequilin on the long column. Alternatively, 95% recovery of the α-estradiol containing little or no β-dihydroequilin can be attained by decreasing the estradiol cut prescribed in the method by 10%. These considerations are applicable to column loads of not more than a few hundred micrograms. Although the limits are not valid for larger loads, the relations are still valid as a guide to the use of the partition system. As indicated in preceding publications, the column is often very useful in purifying relatively large quantities of an estrogen contaminated with small quantities of other closely related estrogens not removable by fractional crystallization.

SUMMARY AND CONCLUSIONS

1. A partition chromatographic system for the separation of certain estrogenic diols consists of NaOH solution fixed on Celite as the immobile phase and benzene as the mobile phase.

2. The effects of alkali concentration, flow-rate, temperature, and degree of packing on the operation of the system are discussed. Temperature effects are very marked and must be regarded when attempting separations.

3. Details are presented of the preparation and analytical use of a short column which permits separation of the estradiols and dihydroequilins as a group from the dihydroequilenins.

4. Similar information is presented for a longer column which also permits separation of the estradiols from α-dihydroequilin for analysis.

5. Theoretical considerations are presented which permit calculation of the actual separations attainable in combination with empirically determined relations between threshold and cut-off volumes.

6. Partition coefficients for β-estradiol and α-dihydroequilin between the mobile and immobile phases have been determined at temperatures from 20 to 35°. These estrogens are shown to behave in reasonable agreement with the partition coefficients and the applied theory.

7. Partition coefficients as determined from the columns are reported for α- and β-estradiols, α- and β-dihydroequilins, and β-dihydroequilenin.

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A Preliminary Report on a Collaborative Assay of Conjugated Estrogen Preparations*

By W. E. GAUNT

Three different procedures for the extraction of conjugated estrogen preparations and three different reagents for the development of specific colors have been used in eleven collaborating laboratories on two different conjugated estrogen preparations in an effort to obtain a reliable method for the rapid determination of the conjugated estrogen content of pharmaceutical products. One of the extraction procedures and one of the reagents were found to offer the most promise for further development.

DURING the last year a very considerable effort has been expended in several laboratories in assessing the value of differing chemical methods for the rapid determination of the conjugated estrogen content of pharmaceutical preparations. Although this assessment is not complete, sufficient progress has been made to justify a report at this time to meet the interest of the many industrial organizations and assay laboratories which have been experiencing difficulties with the conjugated estrogen problem.

The collaborative assay was designed to compare the relative merits of different methods for the extraction of conjugated estrogens from pharmaceutical preparations and different reagents for the development of specific colors. The color reagents and two of the extraction procedures have been in common use in the industry or in the laboratories of the Food and Drug Administration; the third extraction procedure was being developed at the time the collaborative assay was organized.

EXPERIMENTAL

Two different conjugated estrogen preparations, one a powder and the other a tablet sample, were used in the assay, the design of which involved in each of the 11 collaborating laboratories the extraction of three successive weighings of both samples by each procedure employed and the development of color on duplicate aliquots of the extracts using each of the reagents. Color measurements were made with the Beckman spectrophotometer both at the wave length of maximum absorption and at λ420. U. S. P. estrone reference standard was used throughout the assay as the standard with which the powder and the tablet samples were compared. The assay had a basic 3 x 3 x 3 x 3 x 2 x 2 x 11 design involving a total possible number of absorption measurements of 3,564. Not all the collaborating laboratories completed the full design, but sufficient information was obtained on each of the conditions of assay to enable a reasonable statistical analysis to be made.

The measurement of absorption at λ420 was undertaken to provide information on the relative efficiency of the extraction procedures in the elimination of nonestrogenic impurities giving nonspecific or background colors with the reagents and for the determination of a "correction" factor, if any, to be applied in calculating the conjugated estrogen content of the samples. An arbitrary correction made by deducting one-half of the λ420 absorption reading from the λ max. absorption reading was being customarily applied in one laboratory and has been introduced into the U. S. P. XIV monographs, for example, on estradiol tablets and estradiol benzoate injection.

A brief description of the extraction procedures and color reagents used is given below. It is not the intent in this report to give the full details of the manipulations involved in the application of these methods.

Extraction Method A was based on the work of Beall and Grant (1) and involved the extraction of the conjugated estrogens into ethylene dichloride or trichloroethylene containing 1% dicyclohexylamine acetate.

Extraction Method B involved the direct extraction of the conjugated estrogens into alcohol or acetone.

Extraction Method C involved the transformation of the conjugated estrogens into free estrogens by acid hydrolysis, the extraction of the free estrogens into benzene, and purification of the extract by the procedure of Bachman and Pettit (2).

Reagent X was sulfuric acid.

Reagent Y was the Marrian-Kober reagent of phenol and sulfuric acid described by Venning, *et al.* (3).

Reagent Z was the iron-Kober reagent of Haenni (4) containing iron, phenol, and sulfuric acid.

Appreciable arithmetical manipulation of the raw data from the collaborating laboratories had to be performed before an analysis could be made to determine the differences which existed between the extraction procedures, the color reagents, the conjugated estrogen samples, and the collaborating laboratories. The initial statistical analysis was made on absolute absorption measurements equivalent to 50 μg. of U. S. P. estrone, 3 mg. of the powder sample, and 0.05 tablet (five-hundredths of a tablet), calculating these equivalents where necessary from the data submitted.

* Received January 14, 1953. Organized by a subcommittee of the Combined Contact Committees, W. E. Gaunt, Chairman, of the A. D. M. A. and the A. PH. M. A. The collaborating laboratories were: Abbott Laboratories; Ayerst, McKenna & Harrison, Ltd.; the Chemical Division of the Food and Drug Administration; Eli Lilly & Co.; The National Drug Company; Pitman-Moore Co.; E. R. Squibb & Sons; Steroid Laboratories; The Upjohn Co.; and Wyeth, Inc.

TABLE I.—λ MAX. READINGS (AS $D_0 \times 1{,}000$) EQUIVALENT TO 50 μG. OF U. S. P. ESTRONE REFERENCE STANDARD (AVERAGES OF THREE READINGS IN EACH LABORATORY)

Laboratory No.	Reagent X	Reagent Y	Reagent Z
2	341	418	654
3	375	354	675 (720)[a]
4	382	445	572
5	339	365	646
6	393	412	697
7	358	417	619
8A	391	443	702
8B	334	357	530
9	329	417 (360)[a]	622
10	374	438	357[b]
11	...	419	642
Average:	362	408	636
99.7% confidence limits[c]	326–397	360–453	596–674

[a] Figures in parentheses are those obtained by the given laboratory in a second series of assays.
[b] Data for Laboratory 10 on Reagent 2 omitted from the average.
[c] Based on within-laboratory variability.

In Table I is shown absorption at λ max. equivalent to 50 μg. of estrone obtained by each laboratory, using the three-color developing reagents. The data show quite clearly the enhanced absorption given by the iron-Kober reagent and also demonstrates the considerable differences existing between the same type of reagent made in the different laboratories.

Absorption data on the tablet and powder samples as well as on U. S. P. estrone is given in Table II, the figures shown being the averages of the values obtained by all the contributing laboratories. It is again apparent that Reagent Z gives enhanced color development; it is interesting to note that this enhancement is limited to the max. absorption, the λ 420 readings with Reagent Z being of the same order as those obtained with Reagent X.

The ratio of the λ max. to λ420 readings was used to compare the relative efficiency of the extraction procedures in eliminating impurities responsible for nonspecific background colors. These ratios are shown in Table III, which shows that Extraction Method B is the least efficient and that all the extraction procedures are more efficient on the powder than on the tablet samples.

A considerable effort was made to determine whether the λ420 reading contributed to the absorption at λ max. and whether some correction factor would have to be applied on attempting to calculate the conjugated estrogen content of the powder and tablet samples. The most direct approach to this problem was afforded by determining the relationship between the ratio of the λ max. readings on the sample and the U. S. P. estrone standard and the λ420 values on the sample. Using the data from all collaborating laboratories, these calculations showed that, under some of the assay conditions, the λ420 values definitely influenced the λ max. readings, whereas under other conditions, this was not the case. Figures 1 and 2 provide illustrations of such differing conditions, the former showing the lack of dependence of the ratio of the λ max. reading for the sample and the λ max. reading for the standard on the λ420 reading on the sample, using Method A and Reagent Z, while the latter shows the marked dependence of this ratio on the λ420 readings, using Method B and Reagent X. It was similarly found that, using Method C and Reagent Z, the λ max. readings were independent of the λ420 readings.

It was thus determined that under some of the conditions of the assay, a correction would have to be applied in calculating the conjugated estrogen content of the powder or tablet samples. What this correction factor should be in the case of Method B and Reagent X, for example, is not known, but it is certainly not the deduction from the λ max. of one-half of the λ420 readings. Fortunately, it was also determined that with some of the conditions of assay, no correction was needed and that calculation of the conjugated estrogen content of the samples could be made by direct comparison of the λ max. readings on the sample with the λ max. readings on the estrone standard.

Calculation of the conjugated estrogen content of the powder and tablet samples under the conditions of Extraction Methods A and C with Reagent Z are given in Table IV. It is quite apparent that Method C gives lower results than Method A, which is particularly marked with the tablet sample.

It is interesting to note that if a correction factor of one-half of the λ420 reading had been applied in calculating the conjugated estrogen content of the tablet, the content would have appeared to be 1.00 mg./tablet. This result is unacceptably low in view of the fact that both Laboratory 3 and Laboratory 9, using the isolation technique employed by the Food and Drug Administration, obtained values of 1.08 and 1.07 mg./tablet, respectively. Similarly the application of this arbitrary factor to the data on

TABLE II.—AVERAGE ABSORPTION MEASUREMENTS (AS $D_0 \times 1{,}000$) FOR ALL COLLABORATING LABORATORIES

Sample	Method	Reagent X: λ max.	Reagent X: λ420	Reagent Y: λ max.	Reagent Y: λ420	Reagent Z[e]: λ max.	Reagent Z[e]: λ420
U. S. P. Estrone[a]	..	362	39 (10)[d]	408	21 (11)	636	25 (10)
Powder[b]	A	459	164 (9)	438	119 (10)	759	159 (9)
	B	572	325 (7)	457	247 (7)	892	336 (7)
	C	462	160 (5)	444	98 (6)	724	130 (5)
Tablet[c]	A	372	163 (9)	374	149 (10)	595	156 (9)
	B	413	251 (6)	351	224 (6)	710	272 (7)
	C	324	118 (5)	318	79 (6)	510	98 (5)

[a] Absorption values given as equivalent to 50 μg. of estrone.
[b] Absorption values given as equivalent to 3.0 mg. of powder.
[c] Absorption values given as equivalent to 0.05 tablet.
[d] Figures in parentheses show number of laboratories contributing data under the specified conditions.
[e] Data on Reagent Z for Laboratory 10 omitted.

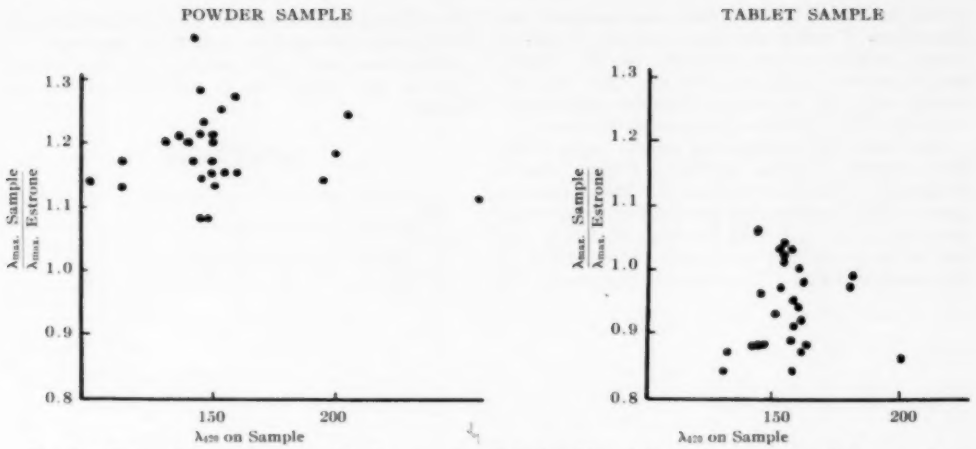

Fig. 1.—Extraction Method A with Reagent Z. Relationship of the ratio $\lambda_{max.}$ sample/$\lambda_{max.}$ estrone to λ_{420} on sample. Ratios calculated from $\lambda_{max.}$ readings equivalent to 3 mg. of powder, 0.05 tablet, and 50 μg. of estrone. λ_{420} readings expressed as D_0 x 1,000.

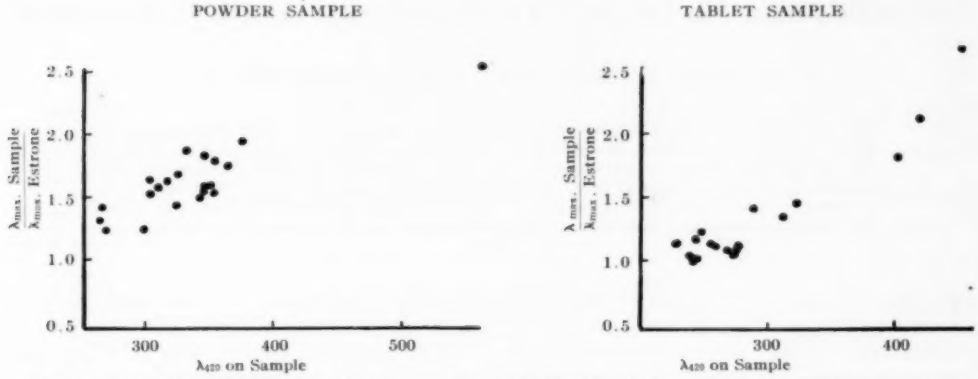

Fig. 2.—Extraction Method B with Reagent X. Relationship of the ratio $\lambda_{max.}$ sample/$\lambda_{max.}$ estrone to λ_{420} on sample. Ratios calculated from $\lambda_{max.}$ readings equivalent to 3 mg. of powder, 0.05 tablet, and 50 μg. of estrone. λ_{420} readings expressed as D_0 x 1,000.

the powder sample would lead to an unacceptably low result.

Work is in progress to determine if the difference between the results obtained by using Extraction Method A and Extraction Method C is due to the destruction of estrogenic materials during the acid hydrolysis. At the moment, it is considered that Extraction Method A with Color Reagent Z gives the best estimate of the conjugated estrogen content of the two samples. This consideration is supported

TABLE III.—RATIO OF ABSORPTION AT λ MAX. AND ABSORPTION AT λ420—AVERAGE VALUES FOR ALL COLLABORATING LABORATORIES

Sample	Method	Reagent X	Reagent Y	Reagent Z[a]
Powder	A	2.80	3.68	4.76
	B	1.76	1.85	2.65
	C	2.88	4.52	5.56
Tablet	A	2.28	2.51	3.82
	B	1.65	1.57	2.61
	C	2.74	4.02	5.20

[a] Data on Reagent Z for Laboratory 10 omitted.

TABLE IV.—CONJUGATED ESTROGEN CONTENT OF POWDER AND TABLET SAMPLES BY LABORATORIES (EACH FIGURE IS AVERAGE OF THREE SEPARATE DETERMINATIONS)

Laboratory	Powder, Mg./Gm. — Method A, Reagent Z	Powder, Mg./Gm. — Method C, Reagent Z	Tablet, Mg./Tablet — Method A, Reagent Z	Tablet, Mg./Tablet — Method C, Reagent Z
2	24.7[a]	23.7[a]	1.11[a]	1.10
3	27.3	25.6	1.29	1.10
4	28.5	27.2	1.34	1.02
5	26.6	...	1.24	...
6	26.4	...	1.21	...
8A	26.7	...	1.24	...
8B	26.2	...	1.37	...
9	27.3	26.9	1.34	1.09
11	27.7	25.7	1.31	1.13
Average	26.7	25.9	1.27	1.08

[a] These results are significantly low.

by the results of duplicate bioassays conducted by Laboratory 2 using the 2-aminopyridine salt of estrone sulfate as the standard; on the powder sample, values of 26.2 and 27.1 mg./Gm. were obtained, while the results on the tablet sample were 1.25 and 1.30 mg./tablet of sodium estrone sulfate.

The variability of the assay on the basis of the data available is about ±10% on a single sample weighing (95% probability limits). This is unsatisfactorily large and further refinements of the assay are now under study with the hope that they will lead to an appreciable increase in the reliability of the assay procedure. It is expected that the details of a reliable method for the rapid determination of the conjugated estrogen content of pharmaceutical preparations, based upon the collaborative work outlined in this report, will be published in the near future.

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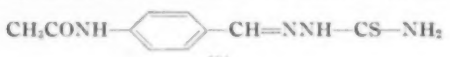
Amithiozone (Tibione) Analogs from Aralkyl Ketones*

By W. LEWIS NOBLES† and J. H. BURCKHALTER‡, §

The antitubercular thiosemicarbazones listed in the literature have been restricted almost exclusively to those derived from aldehydes. In the present report, a group of 18 thiosemicarbazones prepared from aralkyl ketones has been described. The carboxyl or amino group has been introduced into three of the products in an attempt to change their properties or activity. *p*-Chloroacetophenone thiosemicarbazone was found to have four times the *in vitro* effectiveness of 4,4′-diaminodiphenyl sulfone.

THREE years ago, the authors became interested in the synthesis of certain ketonically derived analogs of *p*-acetamidobenzaldehyde thiosemicarbazone (1), the potent antitubercular drug known as amithiozone or Tibione (I), be-

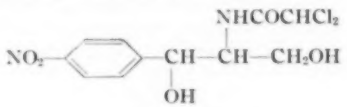


(I)

cause the literature failed to report many derivatives of such origin. Although one laboratory decided that thiosemicarbazones of aldehydes are more active than thiosemicarbazones of ketones, the group failed to list a single ketone derivative (2). Another group described the preparation of more than 100 thiosemicarbazones of aldehydes but only 7 such derivatives of ketones (3). However, a recent report indicates that the thiosemicarbazone of the ketone 4-acetylpyridine appears to have the same order of activity in mice as does amithiozone. (4).

DISCUSSION

All the compounds reported in Table I are ketone thiosemicarbazones. Several (compounds 1, 2, 3, 4, 5, and 6) are obvious variations of amithiozone (I). Others (5, 7, 8, 9, 10, 11, 12, and 13) are related to the antibiotic Chloromycetin (II), while still retaining a similarity to amithiozone. Three



(II)

of the compounds (8, 9, and 14) represent attempts to introduce solubilizing functional groups which might be expected to influence the distribution of the molecule in the host and bacterium.[1] Finally, recalling the intrinsic antibacterial effect of α,β-unsaturated carbonyl compounds (5), it was decided

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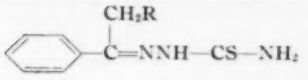
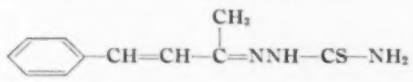
§ The authors wish to thank the American Foundation for Pharmaceutical Education for the financial aid to W. Lewis Nobles which made these studies possible.

[1] After these studies had been initiated, Behnish, Mietzsch, and Schmidt (2) prepared the semicarbazones of certain aldehydes which were substituted with solubilizing groups. There were no comments on the efficacy of this type.

TABLE I.—THIOSEMICARBAZONES



No.[a]	R	Phenyl Substituents	Procedure	Yield, %	M. P.,[b] °C.	Formula	Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
1	H	4-Chloro[c]	A	96	201	$C_9H_{10}ClN_3S$	47.46	47.14	4.42	4.57
2	CH_3	4-Chloro[c]	A	85	176	$C_{10}H_{12}ClN_3S$	49.67	50.01	5.00	5.16
3	H	4-Methoxy[c]	A	94	150	$C_{10}H_{13}N_3OS.H_2O$	49.61	49.59	6.25	6.14
4	H	4-Phenyl[c]	A	90	264	$C_{15}H_{13}N_3S$	66.88	66.80	5.61	5.77
5	H	4-Nitro[d]	A	90	244	$C_9H_{10}N_4O_2S$	45.36	45.87	4.23	4.03
6	H	Replace phenyl with 2-thienyl[e]	A	89	136[r]	$C_7H_9N_3S_2$	42.21	42.20	4.55	4.63
7	α-Dichloroacetamido	4-Nitro[f]	A	88	196	$C_{11}H_{11}Cl_2N_5O_5S$	36.27	36.36	3.06	3.08
8	Amino	4-Nitro[d]	B	80	222	$C_9H_{11}N_5O_2S.HCl$[o]	.....	.....	....	..
9	Dimethylaminomethyl	4-Nitro[g]	B	86	153	$C_{13}H_{17}N_5O_2S.HCl$[p]	.....	.....	....	..
10	Acetoxy	4-Nitro[h]	A	89	212	$C_{11}H_{12}N_4O_4S$	44.60	44.80	4.09	4.20
11	Acetamido	4-Nitro[d]	A	84	240	$C_{11}H_{13}N_5O_3S$	44.73	45.24	4.44	4.77
12	H	3,5-Dinitro[i]	A	90	258	$C_9H_9N_5O_4S$	38.17	38.17	3.21	3.10
13	H	4-(p-Nitrophenyl)[j]	A	77	265	$C_{15}H_{14}N_4O_2S$[q]	57.31	57.77	4.48	4.45
14	α-Carboxymethyl	None[k]	C	82	171	$C_{11}H_{13}N_3O_3S$	52.57	52.47	5.21	5.54



15	...	None[l]	A	93	147	$C_{11}H_{12}N_2S$	.....	.....	..	..
16	...	4-Nitro[i]	A	92	250	$C_{11}H_{12}N_4O_2S$	49.98	50.06	4.57	4.67
17	...	2-Chloro[m]	A	86	185	$C_{11}H_{11}ClN_2S$	52.06	52.50	4.77	4.71
18	...	2,3-Dimethoxy[n]	A	91	188	$C_{13}H_{17}N_3O_2S$	55.89	56.00	6.13	6.16

[a] Compounds 1, 3, 4, 6, 14, and 17 are white; 2, 5, 7, 8, 9, 11, 12, 13, 15, 16, and 18 are various shades of yellow; and 10 is orange.
[b] Melting points are uncorrected.
[c] Intermediate ketones from Eastman Kodak Co.
[d] Ketone obtained through the courtesy of Parke, Davis and Co.
[e] 2-Acetothienone obtained through the courtesy of Socony-Vacuum.
[f] α-Dichloroacetamido-p-nitroacetophenone prepared according to L. M. Long and H. D. Troutman, *J. Am. Chem. Soc.*, **73**, 481(1951).
[g] β-Dimethylamino-p-nitropropiophenone hydrochloride, m. p. 186–189°, prepared according to J. H. Burckhalter and S. H. Johnson, Jr., unpublished report.
[h] p-Nitrophenacyl acetate prepared according to C. Engler and C. Zielke, *Ber.*, **22**, 203(1889).
[i] For ketone, see experimental part.
[j] Ketone prepared according to W. S. M. Grieve and D. H. Hey, *J. Chem. Soc.*, **1933**, p. 968.
[k] β-Benzoylpropionic acid prepared according to "Organic Syntheses," Coll. Vol. II, p. 81.
[l] Thiosemicarbazone previously reported by S. Gheorghui, *Bull. soc. chim.*, [5] **1**, 97(1934).
[m] Supplied by Dr. S. H. Johnson; see ref. 5.
[n] Supplied by Mr. J. R. Campbell; see ref. 5.
[o] Calculated for Cl: 12.24. Found: 12.46.
[p] Calculated for Cl: 10.68. Found: 10.82.
[q] Purified only by successive treatments with hot solvents such as benzene, water, and alcohol.
[r] Ref. 6 gives 148–149°.

to prepare compounds 15, 16, 17, and 18 which might also be classed as vinylogous relatives (5) of compounds 1–6.

All the thiosemicarbazones were prepared by treatment of the ketone with thiosemicarbazide in alcohol-water solution in the presence of a few drops of mineral acid. Usually, fifteen to twenty minutes of heating at steam-bath temperature was sufficient time to gain complete reaction and satisfactory yields. This is in contrast to the period of six to eight hours reported by others (6). In several cases the product precipitated spontaneously when solutions containing the reagents were mixed. In other cases it was necessary, after heating for the required time, to cool the mixture to induce crystallization. With p-chloropropiophenone, the product appeared as a yellow oil after the period of heating, and vigorous agitation induced crystallization.

The intermediate ketones were, in general, prepared by the well-established methods of the literature. Because of the difficulty of obtaining and keeping p-nitrobenzaldehyde, as a result of the oxidizing effect of alkali and air, the best procedure for obtaining p-nitrobenzalacetone was found to be through the nitration of benzalacetone at −20° (7). Another ketone, 3,5-dinitroacetophenone, was prepared by modifying the general method for ketones developed by Walker and Hauser (8).

Biological Results.—The thiosemicarbazones of Table I were submitted to Dr. G. P. Youmans of Northwestern University for *in vitro* screening. Thus far, results of testing of 10 of the 18 compounds have been received: Nos. 1, 3, 4, 5, 6, 11, 12, 14, 15, and 17. p-Chloroacetophenone thiosemicarbazone (No. 1) is four times as active as 4,4′-diaminodiphenyl sulfone, the reference drug. β-Benzoylpropionic acid (No. 14) was devoid of activity at 10 mg. %. The remaining 8 compounds were reported to be active, though less so than compound 1.

While it is realized that a large percentage of compounds which show *in vitro* activity fail to possess *in vivo* effectiveness (9), several of the products of Table I have been submitted for *in vivo* testing.

EXPERIMENTAL[2]

3,5-Dinitroacetophenone.—The following procedure is similar to the general method of Walker and Hauser (8) but differs in several important details. Into a 300-ml. flask equipped with a mechanical stirrer, dropping funnel, and thermometer, 3.9 Gm. (0.11 mole) of magnesium turnings, 0.5 ml. of dry carbon tetrachloride, and 2.5 ml. of absolute alcohol were placed. As soon as the magnesium began to react with the alcohol, 25 ml. of dry chlorobenzene was added rapidly, and the reaction was allowed to proceed to completion.

A solution of 17.6 Gm. (0.11 mole) of diethyl malonate, 12.5 ml. of chlorobenzene, and 10 ml. of absolute alcohol was added to the cooled and stirred mixture at a rate to keep the temperature at about 65°. When the reaction had proceeded to the extent that removal of the cooling bath did not result in a rise in temperature, the mixture was heated slowly to 85° and kept there until the amount of unreacted magnesium became constant (usually about ninety minutes).

The clear, dark solution was cooled to 25°, and a solution of 23.1 Gm. (0.1 mole) of 3,5-dinitrobenzoyl chloride (Eastman) in 63 ml. of xylene was added with stirring and cooling so that the temperature did not exceed 35°. When about half of the acid chloride had been added, a brown gelatinous mass precipitated, and stirring (which was maintained for thirty minutes) became difficult. The flask was then cooled with ice, and a solution of 7 ml. of sulfuric acid in 50 ml. of water was slowly added. The mixture was transferred to a separatory funnel and the chlorobenzene layer was separated and concentrated *in vacuo*. The residue was heated at reflux temperature with a solution of 30 ml. of glacial acetic acid, 4 ml. of concentrated sulfuric acid, and 20 ml. of water for six hours, after which decarboxylation was complete. The mixture was added slowly with stirring to 400 Gm. of cracked ice. The solid product was filtered, washed with water, and then melted under 100 ml. of water and stirred while 12 Gm. of sodium bicarbonate was added. The product was again filtered and treated with 4 Gm. of sodium bicarbonate. After recrystallization from alcohol, 14.8 Gm. (67% yield) of off-white 3,5-dinitroacetophenone was obtained; m. p. 82–83°, reported (10) 82–84°.

***p*-Nitrobenzalacetone.**—To 400 Gm. of concentrated sulfuric acid cooled to 0°, 170 Gm. (1.17 moles) of benzalacetone was slowly added with stirring. By use of an acetone-dry ice bath, the temperature was lowered to −20°, and a nitrating solution containing 90 Gm. (1.18 moles) of concentrated nitric acid (sp. gr. 1.42) in 200 Gm. of concentrated sulfuric acid was added as rapidly as possible without allowing the temperature to rise above −15°. Large amounts of dry ice in the bath allowed rapid addition. Stirring was continued for three hours whereupon the yellow solid was collected on a fritted glass funnel, washed with water, and air dried. After recrystallization from alcohol, 155 Gm. (69% yield) of light yellow product was obtained; m. p. 109–110°, reported (7) 110°.

The *ortho* isomer was not isolated, since no trace of it was visible in the original filtrate of the *para* isomer. However, it has been isolated when the nitration proceeded at higher temperature (5).

[2] Microanalyses by Mr. C. W. Beazley, Skokie, Ill.

ARALKYL KETONE THIOSEMICARBAZONES

Procedure A.—The ketone (0.05 mole) was dissolved in the minimum amount of alcohol. Concurrently, 4.5 Gm. (0.05 mole) of thiosemicarbazide was dissolved in the necessary volume of warm water. To a mixture of the two solutions, first 10 drops of concentrated hydrochloric acid and then 2 Gm. of sodium acetate trihydrate were added. The resulting mixture was heated on the steam bath for fifteen to twenty minutes during which time a heavy precipitate generally formed. The flask was cooled in an ice bath, and its contents were collected, washed with warm water, and dried in the air. Usually, thiosemicarbazide was more water soluble than the product; hence, trituration of the crude product with boiling water served as an excellent method of purification. An analytical sample was obtained by recrystallization from alcohol or diluted alcohol.

Procedure B.—The amino ketone hydrochloride (0.05 mole) was dissolved by warming in diluted alcohol to which a few drops of concentrated hydrochloric acid had been added. At the same time, 4.5 Gm. of thiosemicarbazide was dissolved in a minimum amount of warm water. A mixture of the two solutions was heated for almost twenty minutes. After cooling the solution overnight in an ice chest, the yellow crystals which had formed were collected on a funnel. An analytical sample was obtained by repeated recrystallizations from acidified diluted alcohol.

Procedure C.—A mixture of 8.9 Gm. (0.05 mole) of β-benzoylpropionic acid and 4.1 Gm. of sodium acetate trihydrate was dissolved in 125 ml. of alcohol. This solution was mixed with another containing 4.5 Gm. (0.05 mole) of thiosemicarbazide, 0.5 ml. of concentrated hydrochloric acid, and 100 ml. of warm alcohol. The mixture was heated at reflux temperature for about forty-eight hours. After cooling the solution overnight in an ice chest, the white solid product was collected and air dried. It was recrystallized from alcohol.

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An Evaluation of the Effectiveness of Antiperspirant Preparations Using Frog Membrane and Radioactive Tracer Techniques*,†

By CHIEKO URAKAMI and JOHN E. CHRISTIAN

Astringents have been shown to increase the rate of iodide ion penetration of frog membrane, using radioiodine as a tracer. The increase is proportional to the amount and effectiveness of the astringent. Commercial antiperspirant preparations (liquid and cream) were tested by comparing their relative effect on the penetration of iodide ion when applied side by side on frog skin. Liquid preparations were compared with liquids, and cream preparations were compared with creams. A group of fifteen commercial and research preparations were evaluated by the method described.

EXTENSIVE experimentation during the past four years, using radioactive tracer techniques, on the permeability of frog membrane and the effect of astringents on this permeability has led to the realization that such procedures may be used for comparing the effectiveness of antiperspirant preparations. The technique may be used not only to evaluate the effectiveness of the active ingredients but also to compare the effect of various combinations and formulation ingredients. The need for such a method is apparent to those working in this and related fields.

A rather unusual and unexpected effect of astringents on the permeability of frog membrane by iodide and sodium ions was reported by Lux and Christian (1, 2) who showed that iodide and sodium ion permeability of frog membrane increases after treatment with aluminum salt astringents. Every manner of change in the experimental conditions, without exception, demonstrated this effect. In addition, it was clearly shown that the increased permeability effect is directly proportional to the concentration of the astringents used, which suggested the possibility of using the technique described for comparative evaluations of astringent formulations employed as antiperspirant preparations. The finished formulated preparation, rather than merely the active ingredients, may thus be evaluated by comparison.

EXPERIMENTAL

A modification of the procedure described by Lux and Christian (2) was used to compare a number of commercial liquid and cream antiperspirant preparations. One cream and one liquid were used as standards of comparison. Live, double pithed frogs (*Rana pipiens*) were employed. The cream (250 mg.) chosen as the standard was applied to a 6-cm². area on one side of the back skin and the cream under test (250 mg.) was applied to a similar area on the other side. The same procedure was used for the liquid preparations except that 0.3 cc. each of the standard and the liquid under test were applied separately to a double thickness of gauze 2 x 2.5 cm. in size. The two gauze preparations were mounted on separate pieces of waxed paper, which in turn were attached to adjacent areas of the frog skin. At the end of sixty minutes, the preparations were washed from the skin and the respective skin areas were removed and divided into the separately treated portions. Using No. 8 cotton thread, the two membrane portions were attached (inside out) over the ends of 12-mm. glass tubing cut in lengths 15 cm. long, the ends of which had been slightly flared to prevent slippage of the membrane which was kept moist during the attaching process. Two cubic centimeters of tap water was placed inside each tube which was then lowered (approximately 3 mm.) into a 30-cc. beaker containing 4 cc. of a radioactive iodide solution with an activity of 10,000 c. p. m./0.1 cc.[1] After allowing eighty minutes for equilibrium conditions to be established, 0.1-ml. samples were taken concurrently at four intervals of forty minutes each for activity measurements to determine the extent of iodide ion penetration. The results were expressed by calculating the ratio of standard sample activity to tested sample activity at the four-time intervals. Averaging these four values gave an average ratio for the response of each frog membrane. The values for 7–10 separate frog membranes were averaged for an expression of the final ratio. A typical set of results is given in Table I.

By use of the above procedure, the findings of Lux and Christian (1, 2) were substantiated, the activities of two research samples and seven commercial creams obtained on the open market were compared with a commercial cream, and the activities

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Presented to the Scientific Section, A. PH. A., Philadelphia meeting, August, 1952.

† The isotopes used in this research were granted on allocation from the United States Atomic Energy Commission.

[1] All activities were determined by transferring the desired aliquot to an aluminum cup (3 cm. in diameter) in which was placed a piece of filter paper of the same size held flat with "Duco" household cement. The contents of the cup were then evaporated to dryness and counted under a mica window bell-shaped counter 2.3 mg./cm.² thick. The usual corrections for resolution, background, and decay were made and activities were expressed in counts per minute (c. p. m.).

TABLE I.—TYPICAL RESULTS OF THE COMPARATIVE EFFECT OF ANTIPERSPIRANT PREPARATIONS ON FROG MEMBRANE PENETRATION

Frog No.	Time, Min.[a]	Corrected Activity, C. P. M.[b] Product A	Product B	Ratio, A/B[c]	Av. Ratio
1	80	814	487	1.66	1.76
	120	1280	677	1.89	
	160	1820	949	1.91	
	200	1961	1229	1.51	
2	80	872	441	1.97	1.68
	120	992	568	1.74	
	160	1178	803	1.46	
	200	1565	1005	1.56	

Av. of 8 Experiments: 1.75 ± 0.14

[a] Time the membranes were in contact with the iodide solution.
[b] Radioactivity in counts per minute (c. p. m.) of a 0.1-ml. aliquot from the inner solution.
[c] Calculated by dividing the activity obtained from Product A by that obtained from Product B. Product A was taken as the standard against which all creams were compared. Results indicate that Product A is 1.75 times as effective as Product B. The standard deviation is calculated as follows: S. D. = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE II.—THE COMPARATIVE ASTRINGENT EFFECT OF CREAMS TESTED BY FROG MEMBRANE PENETRATION BY IODIDE ION

Commercial Preparation[a]	No. of Animals	Average Ratio	Standard Deviation
Product A	8	4.15[c]	±0.45
Product B	8	1.75	±0.14
Product C	10	1.14	±0.07
Product D	7	2.18	±0.28
Product E	8	1.49	±0.12
Product F	7	2.82	±0.48
Product G	7	3.77	±0.66
Product G (base only)[d]	8	4.41	±0.67
Sample 1[e]	7	1.83	±0.35
Sample 2[e]	8	2.16	±0.23

[a] The active ingredients in these products included aluminum chlorohydroxide complex, aluminum sulfate, aluminum chloride, aluminum methionate, basic aluminum formate, aluminum sulfonate, and aluminum phenolsulfonate.
[b] Calculated by dividing the activity obtained from the various sample treatments into the activity obtained from Product A determined on the same skin at the same time (see Table I).
[c] This particular value was obtained by comparison of Product A against untreated membrane. Product A increased permeability by a factor of 4.15.
[d] Obtained by extraction with ether.
[e] Antiperspirant research samples.

TABLE III.—THE COMPARATIVE ASTRINGENT EFFECT OF LIQUIDS TESTED BY FROG MEMBRANE PENETRATION BY IODIDE ION

Commercial Preparation	No. of Animals	Average Ratio[a]	Standard Deviation
Product H	8	1.09	±0.14
Product I	8	0.23	±0.03
Product J	7	0.46	±0.07
Product K	8	0.61	±0.10

[a] These liquid products were compared with a commercial liquid preparation (Product L); thus the ratios were calculated by dividing the activity obtained from Product L by the activity obtained from the various liquid treatments determined on the same skin at the same time.

of four commercial antiperspirant liquids obtained on the open market were compared with a commercial liquid preparation. The results of these experiments are tabulated in Tables II and III.

SUMMARY

1. The report of Lux and Christian (1, 2) that astringent agents increase iodide ion penetration of frog membrane in direct proportion to the astringent concentration was verified.

2. A procedure for the comparative evaluation of antiperspirant preparations based on the above findings was developed and applied to 15 different samples, most of which were commercial preparations obtained on the open market.

3. On the basis of the findings, the relative efficiencies of the cream preparations are rated in decreasing order as follows: Products A, C, E, B, Research Sample 1, Research sample 2, Products D, F, G, and Product G base alone.

4. On the basis of the findings, the relative efficiencies of the liquid preparations are rated in decreasing order as follows: Products I, J, K, L, and H.

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The Preparation of N-(Dialkylaminoalkyl) Diacetamides*,†

By DANIEL L. KLAYMAN‡ and PIERRE F. SMITH§

Amides, diamides, and diamines exhibit a variety of pharmacologic properties. Very few N,N-diacylamines have been reported. Accordingly, there has been synthesized a series of five N,N-disubstituted diacetamides of the general formula, $R,R'N(CH_2)_nN(COCH_3)_2$. The methods of preparation and the physical properties of the corresponding hydrochlorides are given.

BECAUSE of the variety of useful pharmacologic properties exhibited by both diamines and amides, consideration was given to the preparation of a series of compounds containing both a tertiary amino group and a diacetamide group and conforming to the general formula, $R,R'N(CH_2)_nN(COCH_3)_2$. Initial work of this type was previously carried out by Mattocks (1).[1]

The main methods reported for the preparation of diacetamide or bisacetamide, the prototype of the desired diamides, involve the reaction of acetic anhydride or acetyl chloride with acetamide, its hydrochloride, or its sodium salt under various conditions (2–6).

It was planned to diacetylate the primary amino group of each member of a series of five diamines conforming to the general formula, $R,R'N(CH_2)_nNH_2$. Acetyl chloride proved too violent for initial acetylations. Consequently the less reactive acetic anhydride was used. In general the desired diamine was refluxed with excess acetic anhydride. Removal of the unreacted acetylating agent was best accomplished by vacuum distillation. Attempts to neutralize the reaction mixtures with aqueous solutions of sodium hydroxide or sodium bicarbonate followed by extraction with benzene proved ineffective because of the extreme water solubility of the oily products. Although solid hydrochlorides could not be prepared from the initial acetylation products, the suspected amides were converted into quaternary ammonium salts by treatment with methyl iodide. Semimicro Kjeldahl determinations [Cole and Parks modification (7)] performed on the methiodide derivatives revealed that the products of the above acetylation procedure were mixtures of mostly primary and some secondary amides.

It was necessary, therefore, to find a more suitable method for introduction of the acetyl group on the amide nitrogen. The addition of anhydrous sodium acetate (8) to the initial reaction mixtures and prolonged heating only increased the yield of the previously mentioned oils, containing mostly primary amides.

The crude monoamide-diamide mixtures were treated with excess acetyl chloride and the reaction mixtures were then heated on a water bath to complete the reaction and to remove as much of the unreacted acetyl chloride as possible. Upon cooling, the crude diacetamide hydrochlorides crystallized. Recrystallization was necessary in all cases. The hydrochloride derivative was anticipated in each reaction with acetyl chloride, attributable to the formation of hydrogen chloride as a by-product and its subsequent combination with the tertiary amino group of the diamide.

The substituted diacetamide hydrochlorides described in Table I are to be tested for their pharmacologic activity.

EXPERIMENTAL

Dimethylaminoethyldiacetamide Hydrochloride.—In a 500-cc. flask fitted with a mechanical stirrer and a reflux condenser, 61.2 Gm. (0.6 mole) of acetic anhydride was allowed to fall dropwise on 17.6 Gm. (0.2 mole) of N,N-dimethylethylenediamine[2] and 4.1 Gm. (0.05 mole) of anhydrous sodium acetate. The mixture was then refluxed for five to six hours and was subsequently distilled under reduced pressure to remove the unreacted acetic anhydride and to isolate the resultant amide-diamide mixture. The crude fraction distilling at 78–145° and 13 mm. pressure weighed 52.9 Gm. and was a yellow, hygroscopic, viscid liquid containing small amounts of acetic anhydride. It was soluble in water and most organic solvents. This liquid was reacted with 31.4 Gm. (0.4 mole) of acetyl chloride, and the reaction

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[2] The authors wish to thank Carbide and Carbon Chemicals Division of Union Carbide and Carbon Corp. and American Cyanamid Co. for their generosity in supplying experimental samples of diamines for use in this work.

TABLE I.—N-(DIALKYLAMINOALKYL) DIACETAMIDE HYDROCHLORIDES

Hydrochloride of	M. P.,[a] °C.	Recrystallized from	Yield, %	Formula	Nitrogen Anal., % Calcd.	Found
Dimethylaminoethyldiacetamide	107	Acetone–dioxane	23.2	$C_8H_{17}N_2O_2Cl$	13.43	13.01
Diethylaminoethyldiacetamide	73	Acetone–dioxane	30.0	$C_{10}H_{21}N_2O_2Cl$	11.83	11.81
Dimethylaminopropyldiacetamide	91	Acetone–dioxane	50.3	$C_9H_{19}N_2O_2Cl$	12.59	12.55
Morpholinoethyldiacetamide	68	Methyl ethyl ketone–glacial acetic acid	18.6	$C_{10}H_{19}N_2O_3Cl$	11.17	11.15
Morpholinopropyldiacetamide	91–92	Methyl ethyl ketone-glacial acetic acid	37.6	$C_{11}H_{21}N_2O_3Cl$	10.58	10.21

[a] All melting points are corrected.

mixture was heated on a water bath in a hood to remove unreacted acetyl chloride. White crystals formed as the product cooled. The dimethylaminoethyldiacetamide hydrochloride was recrystallized by adding approximately 30 cc. of acetone to the material, heating to boiling, slowly adding enough dioxane to effect solution, and chilling the solution at 0–10°. Because of the hygroscopic nature of the crystals of this compound (and of the other members of the series), it was found necessary to dry them in an Abderhalden drying apparatus for a minimum of four hours before analysis.

Diethylaminoethyldiacetamide Hydrochloride.—This compound was synthesized from N,N-diethylethylenediamine using the same molar ratio of reactants in a manner similar to the preparation of dimethylaminoethyldiacetamide hydrochloride. Distillation of the initial acetylation product, at 75–147° and 13 mm., and further acetylation with acetyl chloride gave white platelets.

Dimethylaminopropyldiacetamide Hydrochloride.—This compound was synthesized from N,N-dimethylpropylenediamine by the general method. Distillation of the initial acetylation product, at 90–165° and 14 mm., and further acetylation gave white crystals.

Morpholinoethyldiacetamide Hydrochloride.—This compound was synthesized from N-aminoethylmorpholine by the general method. Distillation of the initial acetylation product, at 75–175° and 13 mm., and further acetylation gave white crystals.

Morpholinopropyldiacetamide Hydrochloride.—This compound was synthesized from N-aminopropylmorpholine by the general method. Distillation of the initial acetylation product, at 76–191° and 15 mm., and further acetylation gave buff-colored crystals.

SUMMARY

The syntheses of dimethylaminoethyldiacetamide hydrochloride, diethylaminoethyldiacetamide hydrochloride, dimethylaminopropyldiacetamide hydrochloride, morpholinoethyldiacetamide hydrochloride, and morpholinopropyldiacetamide hydrochloride have been accomplished by reacting the appropriate N,N-dialkylaminoalkylamines with acetic anhydride, followed by further acylation of the isolated amide-diamide mixtures with acetyl chloride.

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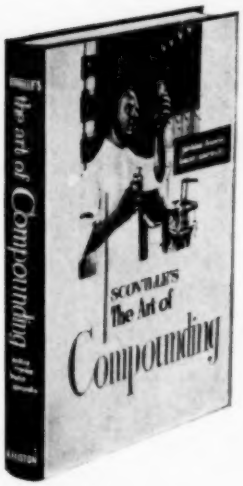

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